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INVENTOR: Ebringer

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DECLARATION OF ALAN EBRINGER UNDER 37 CFR §1.132

I, Alan Ebringer, the sole inventor named in the above-identified patent application and the sole inventor of the subject matter claimed therein, do hereby declare and state as follows:

1. As the sole named inventor of the above-identified patent application, I am intimately familiar with the contents of the application and the subject matter claimed therein. I am the sole inventor of the subject matter disclosed and claimed in the above-identified patent application.

2. I am currently employed as a Professor of Immunology, at King's College London, London, England. I am also currently an Honorary Consultant Rheumatologist at the Middlesex Hospital, University College Hospital School of Medicine, London. I hold an M.D. degree, as well as a B.Sc. degree. I am a Fellow of the Royal College of Physicians-UK (FRCP), a Fellow of the Royal Australasian College of Physicians (FRACP), and a Fellow of the Royal College of Pathologists (FRCPath). I have authored and co-authored numerous papers addressing the etiology, diagnosis, transmission, and treatment of bovine spongiform encephalopathy (BSE).

3. Attached hereto and incorporated herein by reference is a paper that I co-authored entitled "Antibody Responses to *Acinetobacter* spp. and *Pseudomonas aeruginosa* in Multiple Sclerosis: Prospects for Diagnosis Using the Myelin-*Acinetobacter*-Neurofilament Antibody Index." The paper appeared in the journal *Clinical and Diagnostic Laboratory Immunology*, November 2001, pages 1181-1188. I am the corresponding author of the paper.

4. All of the experiments presented in the attached paper were performed either personally by me or at my direction and under my supervision.

5. The experiments presented in the attached paper were conducted to show, in part, that an elevated level of antibodies specific to *Acinetobacter* spp. in a human

subject, as compared to the corresponding antibody level in a control human population, is indicative of multiple sclerosis in the human subject tested.

6. To test the above hypothesis, sera from 26 multiple sclerosis (MS) patients (the human test subject) were obtained. Diagnosis of MS was made according to the Poser criteria, a widely accepted means of diagnosing MS. Sera from 25 normal human subjects (the control group) were also obtained.

7. Cultures of *Acinetobacter* sp. strain 11171, *Acinetobacter* sp. strain 19004, *Acinetobacter junil* 17908, *Acinetobacter lwoffil* 5866, and *Acinetobacter radioresistens* (sp. 12) were obtained from the Public Health Laboratories, Nottingham, UK. *Acinetobacter calcoaceticus* (NCIMB 16904) was obtained from the National Collections of Industrial and Murine Bacteria, Ltd., Aberdeen, Scotland. The cultures were grown in 1 L flasks on an orbital shaker for 2 days at 30°C, in 200 mL of nutrient broth. Flasks were inoculated with two loopfuls of starter culture and left shaking for 6 hours at 37°C. Batch cultures were harvested by centrifugation. Pellets of cells were washed three times in 0.15 M PBS (pH 7.4) and resuspended in 10 mL of PBS. For the ELISA described in paragraph 8 herein, a stock solution of each bacterial suspension was prepared by diluting the suspension in 0.05 carbonate buffer (pH 9.6) to give an OD reading of 0.25 at 540 nm. For SDS-PAGE, the resuspended pellet was ultrasonicated at an amplitude of 12 μm with 30-second bursts and 60-second rest periods (7 cycles). The protein content of the sonicated samples was measured using Bradford's protein assay. The samples were then diluted in sample buffer (0.0625 M Tris-HCl (pH 6.8), 2% SDS, 10% glycerol, 0.001% bromophenol blue) to a protein concentration of 1 μg/μL and heated at 100°C for 3 minutes.

8. Enzyme-linked immunosorbent assays (ELISAs) were prepared by taking aliquots of 200 μL of bacterial suspension, or bovine myelin basic protein (MBP), or neurofilaments (25 μg/mL, Sigma), diluted in a 0.05 M carbonate buffer (pH 9.6), and adsorbing these onto a 96-well flat-bottom polystyrene microELISA plate (Dynatech) overnight at 4°C. Plates were washed three times for 5 min. in PBS containing 0.05% wt/vol Tween 20 ("washing" and "incubation" buffer) and were then blocked with PBS containing 0.1% BSA for 1 hr at 37°C. The washing procedure was repeated and 200 μL of control or test serum, diluted 1 in 200 in incubation buffer, was added to the wells in duplicate and incubated for 1 hr at 37°C. Plates were washed three times in washing buffer, and 200 μL of IgM, IgG, or IgA rabbit anti-human conjugate with horseradish peroxidase (diluted 1 in 500 with incubation buffer) was added and incubated for 1 hr at 37°C. The washing procedure was repeated, and 200 μL of substrate solution was added (substrate solution = 0.5 mg/mL 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) in citrate phosphate buffer, pH 4.1, containing 0.98 mMH_2O_2). The plates were developed in the dark, at room temperature, for 25 min. The reactions were stopped by adding 100 μL of sodium fluoride (2 mg/mL). Absorbances were measured on a microtiter plate reader (Dynatech MR606) at 630 nm. All studies were carried out under blind conditions where the tester did not know which sera were test samples and which sera were controls.

9. The mean OD units of control groups were compared with the mean OD of the 26 MS patients using a one-tail Student's *t*-test, and 95% confidence limits of control groups were calculated. Pearson's correlation coefficient (*r*) was also calculated using the statistical package Prism 3.0 (GraphPad Software).

10. The results of the ELISAs described in paragraph 8 were as follows:
A. Levels of IgA antbodies to *Acinetobacter* sp. strain 11171 ($P < 0.0001$), *Acinetobacter* sp. strain 19004 ($P < 0.0001$), *Acinetobacter junil* 17908 ($P < 0.01$), *Acinetobacter lwoffil* 5866 ($P < 0.0001$), and *Acinetobacter radioresistens* ($P < 0.0001$) in MS patients were significantly higher than those in the healthy control group.
B. Levels of IgG antbodies to *Acinetobacter* sp. strain 11171 ($P < 0.0001$), *Acinetobacter* sp. strain 19004 ($P < 0.0001$), *Acinetobacter junil* 17908 ($P < 0.0001$), *Acinetobacter lwoffil* 5866 ($P < 0.0001$), and *Acinetobacter radioresistens* ($P < 0.0001$) in MS patients were significantly higher than those in the healthy control group.
C. Levels of IgM antbodies to *Acinetobacter* sp. strain 11171 ($P < 0.0001$), *Acinetobacter* sp. strain 19004 ($P < 0.0001$), *Acinetobacter junil* 17908 ($P < 0.0001$), *Acinetobacter lwoffil* 5866 ($P < 0.0001$), and *Acinetobacter radioresistens* ($P < 0.0001$) in MS patients were significantly higher than those in the healthy control group.

11. The results presented in paragraph 10, and in the paper attached hereto and incorporated herein by reference, clearly show, by way of objective scientific evidence, that an elevated level of antibodies specific to microorganisms of the genus Acinetobacter in a human test subject as compared to the corresponding level in known normal individuals, is indicative of MS in the test subject.

12. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application, any patent issuing thereon, or any patent to which this Rule 132 Declaration is directed.

Alan Ebringer 10/4/2002

Alan Ebringer Date

INVENTOR'S PUBLICATION

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Antibody Responses to *Acinetobacter* spp. and *Pseudomonas aeruginosa* in Multiple Sclerosis: Prospects for Diagnosis Using the Myelin-*Acinetobacter*-Neurofilament Antibody Index

L. E. HUGHES,[1] S. BONELL,[1] R. S. NATT,[1] C. WILSON,[1] H. TIWANA,[1] A. EBRINGER,[1]* P. CUNNINGHAM,[1] V. CHAMOUN,[2] E. J. THOMPSON,[2] J. CROKER,[3] ~~AND J. VOWLES~~[3]

Infection and Immunity Group, Division of Life Sciences, King's College London,[1] Department of Neuroimmunology, Institute of Neurology, Queen Square,[2] and Department of Geriatric Medicine, University College Hospital,[3] London, United Kingdom

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Antibody responses to *Acinetobacter* (five strains), *Pseudomonas aeruginosa*, *Escherichia coli*, myelin basic protein (MBP), and neurofilaments were measured in sera from 26 multiple sclerosis (MS) patients, 20 patients with cerebrovascular accidents (CVA), 10 patients with viral encephalitis, and 25 healthy blood donors. In MS patients, elevated levels of antibodies against all strains of *Acinetobacter* tested were present, as well as antibodies against *P. aeruginosa*, MBP, and neurofilaments, but not antibodies to *E. coli*, compared to the CVA group and controls. The myelin-*Acinetobacter*-neurofilament antibody index appears to distinguish MS patients from patients with CVAs or healthy controls. The relevance of such antibodies to the neuropathology of MS requires further evaluation.

Multiple sclerosis (MS) is the most common demyelinating disease of the central nervous system, affecting approximately 80,000 individuals in the United Kingdom and 400,000 in the United States. It is generally thought to be an autoimmune disease that is triggered by an infectious agent, possibly through a molecular mimicry mechanism of induction (1), similar to the situation occurring in the pathology of rheumatic fever (8).

A number of different microbial sequences, including *Pseudomonas* peptides, are capable of activating myelin basic protein (MBP)-specific T-cell clones from MS patients (19), thereby indicating that several microorganisms may carry mimicry epitopes of myelin. This could explain the difficulty of linking the immunopathogenesis of MS to a single microbial agent, suggesting that several such bacteria could be involved in initiating the autoimmune process (6).

It was decided to investigate MS patients for possible immune responses to pathogens that carry molecular mimicry sequences resembling brain components, such as *Acinetobacter* species and *Pseudomonas aeruginosa*. We have identified an amino acid sequence similarity between the encephalitogenic peptide of bovine MBP (5) and a sequence present in 4-carboxymuconolactone decarboxylase of *Acinetobacter calcoaceticus* (3). A similar sequence was also found in γ-carboxymuconolactone decarboxylase of *P. aeruginosa* (Swissprot).

The aims of this study were to determine whether MS patients have elevated levels of antibodies to five different strains of *Acinetobacter* as well as to *P. aeruginosa*, *Escherichia coli*, MBP, and neurofilaments, compared to cerebrovascular-accident (CVA) patients and control healthy blood donors. A further study was carried out, incorporating an inflammatory non-MS disease control, viral encephalitis, and a new set of control subjects, to assess whether MS patients have elevated levels of immunoglobulin G (IgG) antibodies to *A. calcoaceticus* 16904.

MATERIALS AND METHODS

Serum samples. Sera from 26 MS patients (nine males and 17 females; mean age, 42 years; range, 29 to 55 years) were obtained from the Institute of Neurology at the Hospital for Nervous Diseases, London, United Kingdom. Diagnosis was made according to the Poser criteria (13). Benign MS patients are designated by infrequent exacerbation with full recovery. Relapsing remitting MS patients are those patients who have more frequent exacerbations followed by partial or complete remission. Serum samples were obtained from relapsing remitting patients during remission and exacerbation. Secondary progressive MS patients are defined as patients who continue to deteriorate without remission following an initial relapsing remitting course of disease. Primary progressive MS is defined as continuous deterioration without remission from the onset of disease. The clinical features of the MS patients are summarized in Table 1. In addition, serum samples were obtained from 20 patients in the Department of Geriatric Medicine at University College Hospital, who had suffered from a unilateral hemiplegia due to a CVA (10 males and 10 females; mean age, 80.5 years; range, 69 to 94 years) and 10 patients with viral encephalitis (eight males and two females; mean age, 38 years; range, 3 to 66 years) attending the National Hospital for Neurology and Neurosurgery. Sera from 25 subjects attending the London Blood Donor services were used as healthy controls (12 males and 13 females; mean age, 40.6 years; range, 22 to 67 years). A further set of sera from 29 healthy control subjects attending the London Blood Donor services was used in the viral encephalitis study (15 males and 14 females; mean age, 43 years; range, 19 to 66 years).

Preparation of bacteria. Cultures of *Acinetobacter* sp. strain 11171, *Acinetobacter* sp. strain 19004, *Acinetobacter junii* 17908, *Acinetobacter lwoffii* 5866 and *Acinetobacter radioresistens* (sp12) were provided by the Public Health Laboratory, Nottingham, United Kingdom. *A. calcoaceticus* (NCIMB 16904) was obtained from the National Collections of Industrial and Murine Bacteria Ltd. (Aberdeen, Scotland). The Department of Microbiology at King's College London provided *P. aeruginosa* (NCTC 8203) and *E. coli* (NCTC 9002).

Cultures were grown in 1-liter flasks on an orbital shaker for 2 days at 30°C for all *Acinetobacter* strains and at 37°C for *P. aeruginosa* and *E. coli* in 200 ml of nutrient broth (25 g/liter; Oxoid). Flasks were inoculated with two loopfuls of starter culture and left shaking for 6 h at 37°C. Batch culture cells were harvested by centrifugation at 4,000 rpm for 20 min at less than 10°C (six 250-ml aliquots; Beckman JA-20 rotor). Pellets of cells were washed three times in 0.15 M

* Corresponding author. Mailing address: Infection and Immunity Group, Division of Health and Life Sciences, King's College London, 150, Stamford St., London SE1 9NN, United Kingdom. Phone: 020-7848-4302. Fax: 020-7848-4500. E-mail: alan.ebringer@kcl.ac.uk.

TABLE 1. MS patient clinical data

Laboratory no.	Sex[a]	Age (yr)	Diagnosis[b]	EDSS[c]	Disease duration (yr)
MS 006	M	53	B	3.0	20
MS 028	F	54	B	NA[d]	NA
MS 033	F	NA	B	NA	NA
MS 051	F	55	B	1.0	25
MS 054	F	49	B	3.0	17
MS 080	F	49	B	1.0	20
MS 007	F	38	RR	2.5	10
MS 042	F	31	RR	3.5	NA
MS 084	F	50	RR	5.5	8
MS 095	F	32	RR	6.5	3
MS 017	F	53	ARR	4.0	10
MS 019	F	29	ARR	4.0	1
MS 030	F	31	ARR	4.0	7
MS 037	F	34	ARR	5.0	7
MS 087	M	40	Transitional	5.0	9
MS 016	M	49	PP	5.5	5
MS 056	M	41	PP	8.0	1
MS 062	M	31	PP	7.0	10
MS 065	M	43	PP	8.0	8
MS 091	M	47	PP	7.5	23
MS 092	F	44	PP	8.5	6
MS 102	F	47	PP	8.0	26
MS 020	M	31	2P	8.0	5
MS 040	F	33	2P	8.0	2
MS 055	M	51	2P	8.0	10
MS 104	F	38	2P	6.5	4

[a] Abbreviations: M, male; F, female.
[b] Abbreviations: B, benign; RR, relapsing remitting; ARR, acute relapsing remitting; PP, primary progressive, 2P, secondary progressive.
[c] EDSS, expanded disability status scale.
[d] NA, not available.

phosphate-buffered saline (PBS) (pH 7.4) and finally resuspended in 10 ml of PBS. For the enzyme-linked immunosorbent assay (ELISA) a stock solution of the bacterial suspension was prepared by diluting in 0.05 M carbonate buffer (pH 9.6) to give an optical density (OD) reading of 0.25 at 540 nm on a spectrophotometer (Dynatech MR606), which had previously been determined as the optimum concentration (15). For sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis, the resuspended pellet was ultrasonicated at an amplitude of 12 μm with 30-s bursts and 60-s rest periods (seven cycles). The protein content of the sonicated samples was measured using Bradford's protein assay (2). The sample was then diluted in sample buffer (0.0625 M Tris-HCl [pH 6.8], 2% SDS, 10% glycerol, 0.001% bromophenol blue) to a protein concentration of 1 μg/μl and heated at 100°C for 3 min.

ELISA. ELISAs were carried out as previously described (9). Briefly, aliquots of 200 μl of bacterial suspension or bovine MBP or neurofilaments (25 μg/ml; Sigma), diluted in a 0.05 M carbonate buffer (pH 9.6), were adsorbed onto a 96-well flat-bottom polystyrene microELISA plate (Dynatech) overnight at 4°C. Plates were washed three times for 5 min in PBS containing 0.05% (vol/vol) Tween 20 (Sigma) (washing and incubation buffer) and were blocked with PBS containing 0.1% bovine serum albumin (Sigma) for 1 h at 37°C. The washing procedure was repeated, and 200 μl of test or control serum, diluted 1 in 200 in incubation buffer, was added to the wells in duplicate and incubated for 1 h at 37°C. Plates were washed three times in washing buffer, and 200 μl of IgM, IgG, or IgA rabbit anti-human conjugate with horseradish peroxidase (Dako), diluted 1 in 500 in incubation buffer, was added and incubated for 1 h at 37°C. The washing procedure was repeated, and 200 μl of substrate solution—2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) (0.5 mg/ml; Sigma) in citrate phosphate buffer, pH 4.1, containing 0.98 mM H_2O_2 (Sigma)—was added to each well. The plates were developed in the dark, at room temperature, for 25 min. The reaction was stopped by addition of 100 μl of sodium fluoride (2 mg/ml; Sigma) solution. Absorbances were measured on a microtiter plate reader (Dynatech MR606) at 630 nm. All studies were carried out under code, in that the tester did not know which sera were test or control.

Triplicate ELISA studies measuring total immunoglobulins (IgG, IgM, and IgA) against *A. radioresistens*, *Acinetobacter* sp. strain 11171 and *P. aeruginosa* were carried out in serial doubling dilutions using a high- and low-titer MS serum and a control sample (M39).

Statistical analysis. The mean OD units of control groups (CVA and healthy blood donors) were compared with the mean OD of the 26 MS patients, using a one-tail Student's *t* test, and 95% confidence limits of control groups were calculated. Pearson's correlation coefficient (*r*) was also calculated using the statistical package Prism3.0 (GraphPad Software).

Western blot analysis. Electrophoresis was performed according to the method of Laemmli (10). Briefly, prepared samples (10 μl) and a set of molecular weight markers (5 μl) (RPN800; Amersham) were loaded and run on a 10% SDS-polyacrylamide gel at 100 V for 2 h in electrolyte buffer (0.025 M Tris, 0.2 M glycine, and 0.1% SDS, pH 8.3) and the gel was transferred to nitrocellulose membrane at 30 V for 16 h in transfer buffer (20 mM Tris, 0.15 M glycine, pH 8.3, containing 20% [vol/vol] methanol) (Protean III cell; Bio-Rad Laboratories). All incubation steps were carried out at room temperature with gentle shaking. After transfer the membrane was cut into individual strips and incubated in 2% Marvel–Tris-buffered saline (TBS) for 2 h. The strips were washed five times for 5 min in TBS-Tween and then individually incubated with patient or control serum for 2 h. Serum was diluted at 1 in 200 in 2% Marvel–TBS. The strips were then washed as before and incubated for 2 h with rabbit anti-human immunoglobulin (IgG, IgM, and IgA) conjugate with horseradish peroxidase (Dako), diluted 1 in 1,000 in 2% Marvel–TBS. Strips were washed and developed using a chemiluminescent detection assay (Amersham).

RESULTS

IgA anti-*Acinetobacter* antibodies. Levels of IgA antibodies to *Acinetobacter* sp. strain 11171 ($P < 0.0001$), *Acinetobacter* sp. strain 19004 ($P < 0.0001$), *A. junii* 17908 ($P < 0.01$), *A. lwoffii* 5866 ($P < 0.0001$), and *A. radioresistens* ($P < 0.0001$) in MS patients were significantly higher than those in the healthy control group.

Levels of IgA to *Acinetobacter* sp. strain 11171 ($P < 0.0001$), *A. lwoffii* 5866 ($P < 0.0001$), and *A. radioresistens* ($P < 0.0001$) were also shown to be significantly elevated in MS patients compared to CVA patients. No significant difference between MS and CVA patients was seen for IgA antibodies to either *Acinetobacter* sp. strain 19004 or *Acinetobacter* sp. strain 17908.

Significantly elevated levels of IgA to *Acinetobacter* sp. strain 19004 ($P < 0.0001$) and *A. junii* 17908 ($P < 0.001$) were observed in CVA patients compared to controls. Levels of IgA antibody to *Acinetobacter* sp. strain 11171 were shown to be significantly higher in controls ($P < 0.001$) than in CVA patients. No other significant differences were seen in the levels of IgA antibodies to *A. lwoffii* 5866 or *A. radioresistens* between the control and CVA groups (Fig. 1a to e).

Furthermore, when patients were divided into different MS groups, secondary progressive patients were shown to have significantly elevated levels of IgA antibodies to *Acinetobacter* sp. strain 11171 compared to relapsing remitting MS ($P < 0.001$), benign MS ($P < 0.02$), acute relapsing remitting MS ($P < 0.02$), and primary progressive MS ($P < 0.05$) patients. Primary progressive patients were also shown to have significantly elevated levels of IgA to *Acinetobacter* sp. strain 11171 compared to relapsing remitting patients ($P < 0.01$) (see Fig. 3a).

IgG anti-*Acinetobacter* antibodies. Levels of IgG antibodies to *Acinetobacter* sp. strain 11171 ($P < 0.0001$), *Acinetobacter* sp. strain 19004 ($P < 0.0001$), *A. junii* 17908 ($P < 0.0001$), *A. lwoffii* 5866 ($P < 0.0001$), and *A. radioresistens* ($P < 0.0001$) were

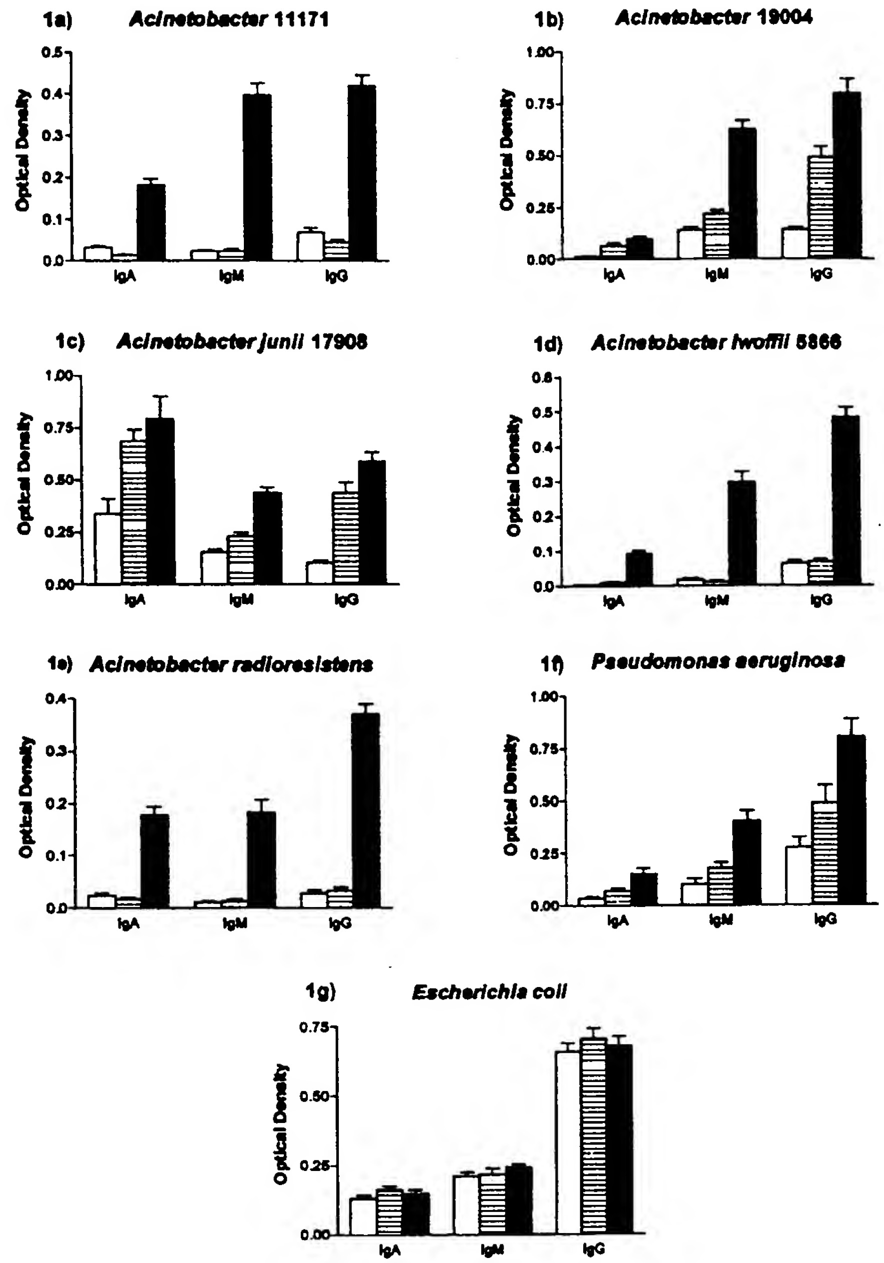

FIG. 1. Levels of IgA, IgM, and IgG antibodies (mean + standard error [error bars]) to *Acinetobacter* sp. strain 11171 (a), *Acinetobacter* sp. strain 19004 (b), *A. junii* 17908 (c), *A. lwoffii* 5866 (d), *A. radioresistens* (e), *P. aeruginosa* (f), and *E. coli* (g), in sera from 26 MS patients, 20 CVA patients, and 25 healthy blood donors. Symbols: □, controls; ■, CVA; ■, MS.

significantly elevated in MS patients compared to healthy controls.

IgG antibodies to *Acinetobacter* sp. strain 11171 ($P < 0.0001$), *Acinetobacter* sp. strain 19004 ($P < 0.01$), *A. junii* 17908 ($P < 0.05$), *A. lwoffii* 5866 ($P < 0.0001$), and *A. radioresistens* ($P < 0.0001$) were significantly higher in MS patients than in CVA patients.

Significantly elevated levels of IgG antibodies to *Acinetobacter* sp. strain 19004 ($P < 0.0001$) and *A. junii* 17908 ($P < 0.0001$) were found in CVA patients compared to healthy

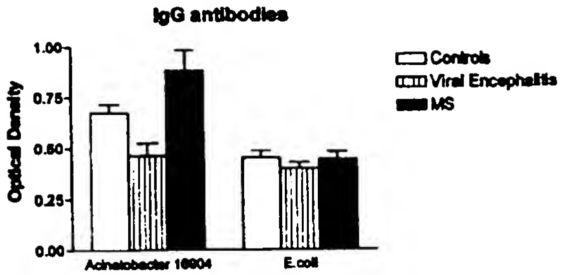

FIG. 2. IgG antibody levels (mean + standard error [error bars]) to *A. calcoaceticus* 16904 and *E. coli* in sera from 26 MS patients, 10 viral encephalitis patients, and 29 healthy blood donors.

controls. However, there was no significant difference in the level of IgG antibodies to *Acinetobacter* sp. strain 11171, *A. lwoffii* 5866, and *A. radioresistens* between the CVA patients and healthy controls (Fig. 1a to e).

A further study, measuring IgG antibody levels to *A. calcoaceticus* 16904 demonstrated a significant elevation in sera from MS patients compared to levels in sera from viral encephalitis patients ($P < 0.02$) and healthy blood donor controls ($P < 0.05$) (Fig. 2).

IgM anti-*Acinetobacter* antibodies. Levels of IgM antibodies to *Acinetobacter* sp. strain 11171 ($P < 0.0001$), *Acinetobacter* sp. strain 19004 ($P < 0.0001$), *A. junii* 17908 ($P < 0.0001$), *A. lwoffii* 5866 ($P < 0.0001$), and *A. radioresistens* ($P < 0.0001$) were significantly higher in MS patients than in the healthy control group.

Levels of IgM antibody to *Acinetobacter* sp. strain 11171 ($P < 0.0001$), *Acinetobacter* sp. strain 19004 ($P < 0.0001$), *A. junii* 17908 ($P < 0.0001$), *A. lwoffii* 5866 ($P < 0.0001$), and *A. radioresistens* ($P < 0.0001$) were also shown to be significantly elevated in MS patients compared to CVA patients.

Significantly elevated levels of IgM antibodies to *Acinetobacter* sp. strain 19004 ($P < 0.001$) and *Acinetobacter* sp. strain 17908 ($P < 0.001$) were found in CVA patients compared to healthy controls. No significant differences were seen in the level of IgM antibodies to *Acinetobacter* sp. strain 11171, *A. lwoffii* 5866, and *A. radioresistens* between the CVA and control groups (Fig. 1a to e).

Antibodies to *P. aeruginosa*. Levels of IgA antibodies to *Pseudomonas* were shown to be significantly higher in MS patients than in the control group ($P < 0.001$) and CVA patients ($P < 0.05$). There was also a significant elevation in the level of IgA anti-*Pseudomonas* antibodies in the CVA group ($P < 0.05$) compared to controls.

Elevated levels of IgG antibodies to *Pseudomonas* were also observed in MS sera compared to controls ($P < 0.0001$) and CVA patients ($P < 0.05$). Furthermore, there was also a significant elevation in the level of IgG antibodies to *Pseudomonas* ($P < 0.05$) in the CVA group compared to controls.

IgM antibody levels to *Pseudomonas* were also shown to be elevated in MS patients compared to controls ($P < 0.0001$) and CVA patients ($P < 0.001$). There was no significant difference in the level of IgM antibodies to *Pseudomonas* between the two control groups (Fig. 1f).

Antibodies to *E. coli*. No significant differences in the levels of IgA, IgM, or IgG anti-*E. coli* antibodies were observed between any of the groups tested (Fig. 1g and 2).

Antibodies to brain components. (i) MBP. Significantly elevated levels of IgA to MBP were observed in MS sera compared to controls ($P < 0.0001$) and CVA sera ($P < 0.001$). However, no significant difference was observed between the control and CVA group, when measuring IgA antibody levels.

Increased levels of anti-MBP IgG antibodies were also seen in MS sera compared to controls ($P < 0.0001$) and CVA patients ($P < 0.01$), and MS patients showed significantly higher levels of IgM antibodies to MBP than those seen in controls ($P < 0.0001$) and CVA patients ($P < 0.0001$). There were also increased levels of anti-MBP IgM and IgG antibodies in CVA patients compared to controls ($P < 0.0001$) (Fig. 3c).

(ii) Neurofilaments. Levels of IgA antibody to neurofilaments were shown to be significantly higher in MS patients than in controls ($P < 0.01$) and CVA patients ($P < 0.0001$). No significant difference between the control and CVA groups was seen in the level of antineurofilament IgA antibodies.

MS patients also showed significantly elevated IgG antineurofilament antibodies compared to the controls ($P < 0.0001$) and CVA patients ($P < 0.01$). The level of IgG antibodies was significantly increased in CVA patients compared to controls ($P < 0.0001$).

Significantly elevated levels of IgM to neurofilaments were observed in MS sera compared to those from healthy controls ($P < 0.0001$) and CVA patients ($P < 0.0001$). There was no significant difference between the control and CVA groups in the levels of antineurofilament IgM antibodies (Fig. 3d).

No significant differences in the levels of IgA, IgM, or IgG antibodies to *A. radioresistens*, *A. lwoffii* 5866, *A. junii* 17908, *Acinetobacter* sp. strain 19004, *P. aeruginosa*, MBP and neurofilaments were found between the different groups of MS patients (Fig. 3).

Measurement of serial dilutions. Levels of total immunoglobulin (IgG, IgM, and IgA) to *A. radioresistens* (Fig. 4a), *Acinetobacter* sp. strain 11171 (Fig. 4b), and *P. aeruginosa* (Fig. 4c) are shown for a high- and low-titer MS serum and a control sample (M39).

For each antigen tested, the high-titer and low-titer MS serum reacted with a dilution of up to 1/6,400 and 1/3,200, respectively, whereas the control serum gave lower readings. The coefficient of variation was less than 10% for all ELISA tests carried out.

Western blot analysis. The specificity of the serum antibodies was evaluated by Western blotting using *A. radioresistens*, *Acinetobacter* sp. strain 11171, and *P. aeruginosa* antigens. The high-titer MS serum (MS080) showed prominent reactivity to a 15- to 28.6-kDa region of both *A. radioresistens* and *Acinetobacter* sp. strain 11171, and a further band of reactivity can be seen with a 9.1-kDa protein of *Acinetobacter* sp. strain 11171. The control serum (M39) showed much weaker reactivity to proteins of *Acinetobacter* sp. Reactivity to a number of proteins of *P. aeruginosa* can be seen in both the MS and control patients. The MS patient demonstrated prominent reactivity to a 78-kDa protein and an 8.5-kDa protein of *P. aeruginosa* (Fig. 5).

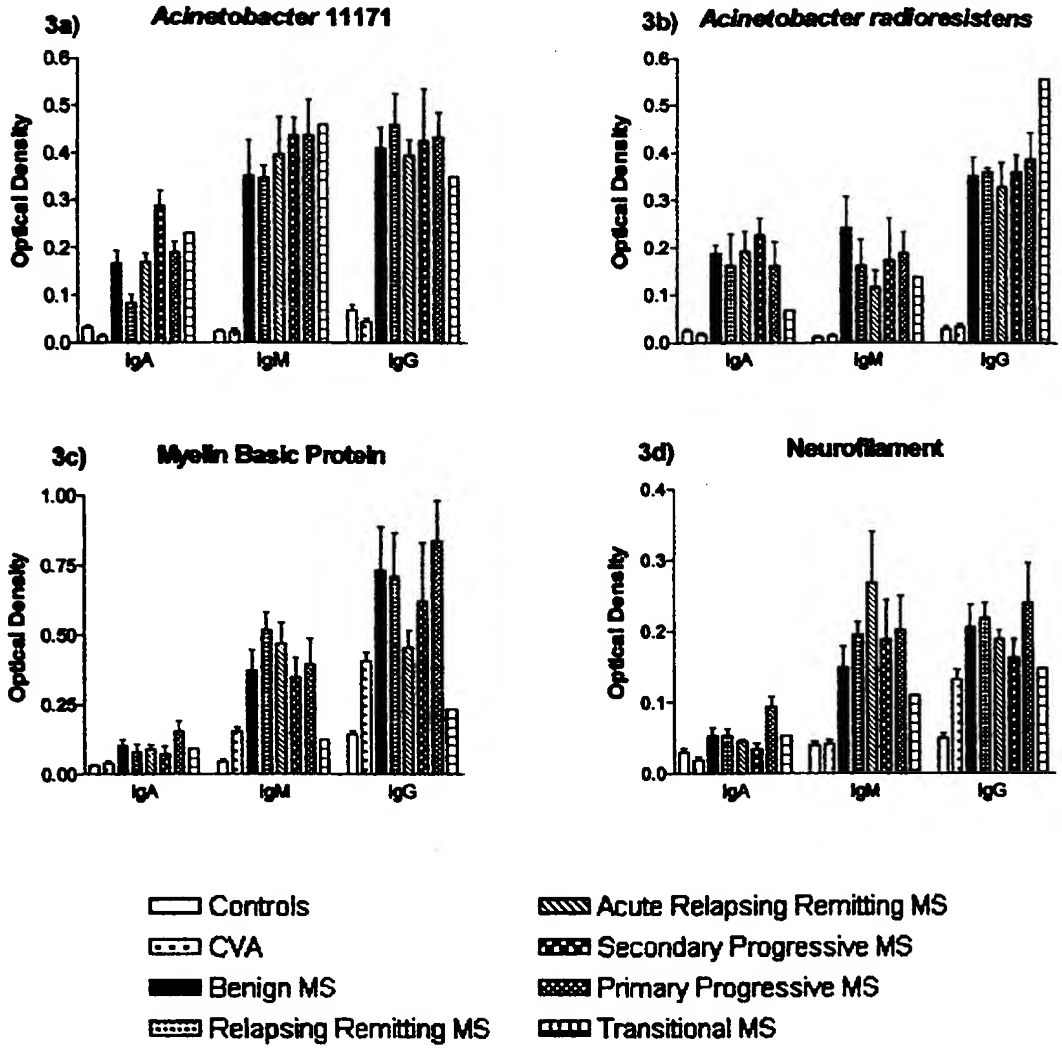

FIG. 3. Levels of IgA, IgM, and IgG antibody (mean + standard error [error bars]) to *Acinetobacter* sp. strain 11171 (a), *A. radioresistens* (b), MBP (c), and neurofilaments (d) in sera from 26 MS patients, 20 CVA patients, and 25 healthy blood donors.

Correlation coefficient analysis. The correlation coefficient (r) was calculated between all strains of *Acinetobacter* tested and *P. aeruginosa*. There was a significant positive correlation between the IgM levels of *A. junii* 17908 and *P. aeruginosa* ($r = +0.831$; $P < 0.0001$). A significant positive correlation was also seen between the IgM levels of *Acinetobacter* sp. strain 19004 and *P. aeruginosa* ($r = +0.819$; $P < 0.0001$). This was also the case for levels of IgA antibodies between these two organisms ($r = +0.407$; $P < 0.01$). Both *Acinetobacter* sp. strain 19004 and *A. junii* 17908 also showed significant positive correlations with both MBP and neurofilaments, with regards to IgM and IgG levels ($P < 0.0001$). A significant positive correlation was seen between the IgM levels of *P. aeruginosa* and both MBP ($r = +0.851$; $P < 0.0001$) and neurofilaments ($r = +0.693$; $P < 0.0001$). A significant positive correlation was also seen between the IgA ($r = +0.399$; $P < 0.05$), IgM ($r = +0.663$; $P < 0.001$) and IgG ($r = +0.772$; $P < 0.0001$) levels of MBP and neurofilaments.

MAN index. The myelin-*Acinetobacter*-neurofilament (MAN) index was calculated using the results from the IgG antibody assays. The formula used was as follows: (MBP OD $\times$ 10) (*Acinetobacter* strain OD/*Pseudomonas* OD $\times$ 10) (neurofilament OD $\times$ 10). All MS patients had values above the 99.9% confidence limit of the controls when the MAN index was calculated using *A. lwoffii* 5866, *Acinetobacter* sp. strain 11171, *A. radioresistens*, *Acinetobacter* sp. strain 19004, and *A. junii* 17908. Only 88.5% of MS patients were shown to have a MAN index above the 99.9% confidence limit, when calculating with *P. aeruginosa* antibodies. In all cases only one control was shown to lie above the 99.9% confidence limit. Seven CVA

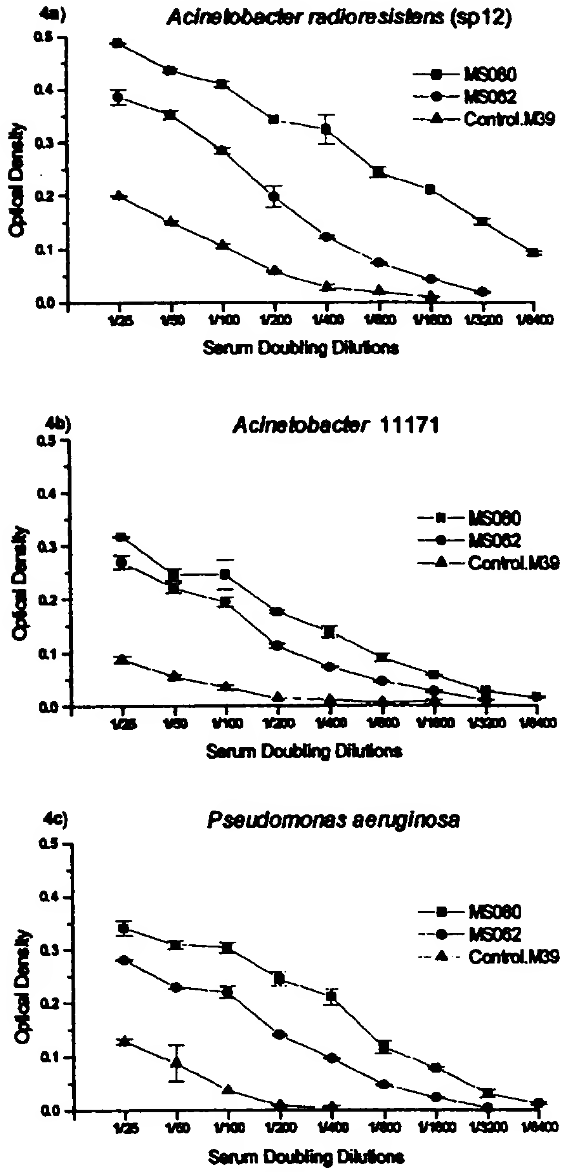

FIG. 4. Serial doubling dilutions (mean ± standard error [error bars]) of high- and low-titer MS sera and a control sample against *A. radioresistens* (a), *Acinetobacter* sp. strain 11171 (b), and *P. aeruginosa* (c).

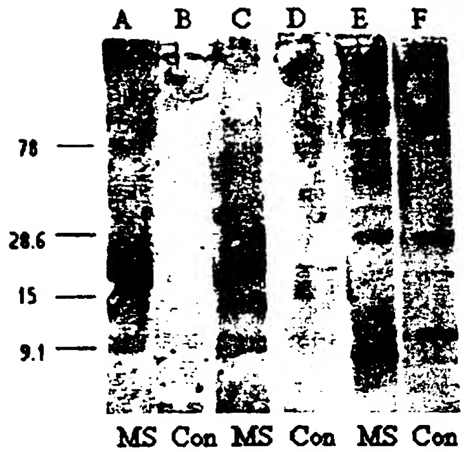

FIG. 5. Immunoblotting antigenic bands developed with serum samples from an MS patient (MS080) (MS) and a control healthy blood donor (M39) (Con) against *A. radioresistens* (lanes A and B), *Acinetobacter* sp. strain 11171 (lanes C and D), and *P. aeruginosa* (lanes E and F).

patients were shown to have values above the 99.9% confidence limit when the MAN index was calculated using *Acinetobacter* sp. strain 11171. For all other strains of *Acinetobacter* and *P. aeruginosa* more than 12 CVA patients had MAN indices above the 99.9% confidence limit. No significant differences were seen in the MAN indices between the different MS groups. Interestingly, when calculating the MAN index using *A. radioresistens* all MS patients could be distinguished from the two control groups. When removing the *Acinetobacter* parameter, the MAN index could distinguish MS patients from healthy controls but not from the CVA group (Fig. 6). When the MAN index was calculated with IgA and IgM antibodies, the differences between the MS patients and control groups were not as significant (data not shown).

DISCUSSION

Elevated levels of IgA, IgM, and IgG directed against *Acinetobacter* sp. strain 11171, *A. lwoffii* 5866, and *A. radioresistens* were found in the sera from MS patients compared to CVA patients or healthy controls. A similar observation was seen for *Acinetobacter* sp. strain 19004 and *Acinetobacter* sp. strain 17908, although there were no significant differences in the levels of IgA antibodies between the MS and CVA groups. A significant increase in levels of IgG to *A. calcoaceticus* 10694 was also shown in MS patients compared to healthy controls and viral encephalitis patients. Primary and secondary progressive MS patients tended to have higher levels of IgA antibodies to *Acinetobacter* sp. strain 11171 compared to other MS groups.

Molecular mimicry appears to be involved in MS (1, 7), but efforts to identify an etiological agent have not been successful. Seven viral and one bacterial peptides have been found to activate three of seven T-cell clones specific to MBP (amino acids 85 to 99) isolated from MS patients. The bacterial peptide identified was from phosphomannomutase protein in *P. aeruginosa* (19). In this study we have looked at potential respiratory pathogens, including *Acinetobacter* and *P. aeruginosa*, which carry sequences that mimic brain components. An amino acid sequence homology between a known encephalitogenic myelin peptide (5) and the enzyme 4-carboxymuconolactone decarboxylase has been found in both *Acinetobacter* spp. and *P. aeruginosa* (3).

Autoantibodies to neuronal components, including MBP (17), myelin oligodendrocyte protein (14), myelin-associated glycoprotein (16), proteolipid protein (18), and neurofilaments

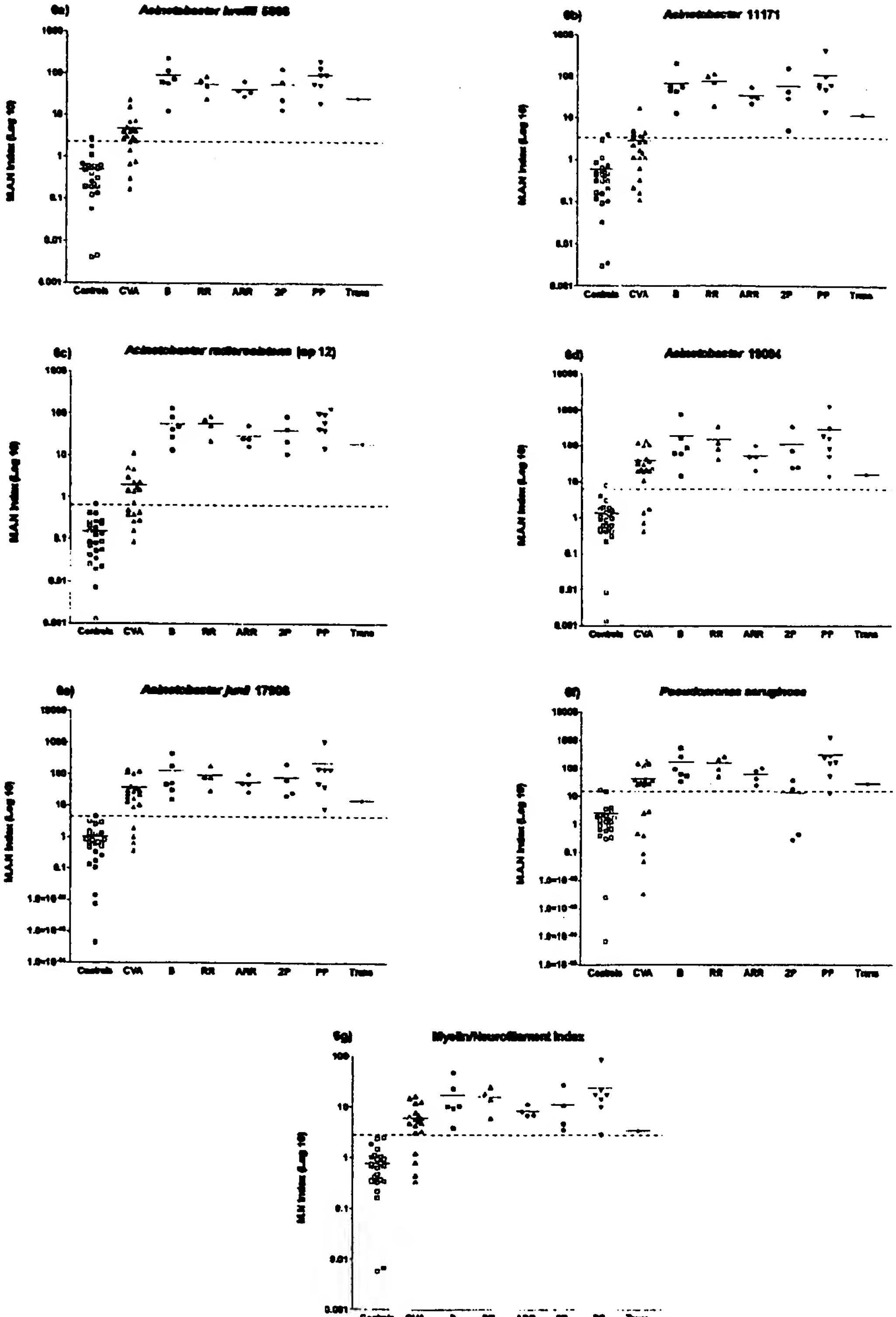

FIG. 6. MAN index, calculated as follows: (MBP OD × 10) (*Acinetobacter* OD/*Pseudomonas* OD × 10) (neurofilament OD × 10) expressed in $\log_{10}$, in sera from 26 MS patients (B, benign; RR, relapsing remitting; ARR, acute relapsing remitting; 2P, secondary progressive; PP, primary progressive; Trans, transitional), 20 CVA patients, and 25 healthy blood donors. The dashed line represents the 99.9% confidence limit of controls.

(11) have previously been reported in MS. IgG antimyelin antibodies, but to a lesser extent IgM and IgA, have also previously been demonstrated in 88% of sera from healthy controls by indirect immunofluorescence assays (4). Increased levels of IgA, IgM, and IgG to both bovine MBP and neurofilaments acting as heterospecific antigens were observed in MS patients compared to controls and the CVA group. There is 92% homology between bovine and human MBP, but almost complete amino acid identity over the most immunogenic epitopes (5). If the presence of anti-MBP and antineurofilament autoantibodies occurs as a result of previous brain damage, antibodies to these brain components should also be found in CVA patients as well as MS patients. However, the results of this study indicate that the MS sera but not the CVA sera had significantly elevated levels of these autoantibodies compared to controls. The pathology of MS is usually thought of as being confined to the white matter, but the observation that autoantibodies to neurofilaments were seen in MS patients and not controls indicates that gray matter of the brain may also be involved in the disease. This confirms previous observations indicating the presence of gray matter pathology in MS patients using magnetic resonance imaging (12) and studies demonstrating the presence of antibodies to the 69-kDa neurofilament protein (11).

We have used our results from the ELISAs measuring IgG antibodies against MBP, *Acinetobacter*, *P. aeruginosa*, and neurofilaments to obtain a MAN antibody index. This parameter made it possible for us to identify all the MS patients, and therefore it could offer the possibility of a diagnostic test, but prospective studies will be required to establish the validity of the MAN index.

The agent responsible for autoantibodies in MS is still unclear, but this study does show that MS patients have probably been exposed to *Acinetobacter* spp. and *P. aeruginosa*. It is likely that infection with these microorganisms, which carry epitopes resembling brain antigens, could lead to activation of auto-reactive cells and production of autoantibodies, which may cause tissue damage and thus lead to the development of a neurological autoimmune disease, such as MS. However, it must be considered that in the ELISA we are detecting anti-MBP antibodies that are cross-reacting with *Acinetobacter* spp. Longitudinal studies are necessary to determine whether exposure to *Acinetobacter* antigens precedes or follows pathological changes. Further work is required to evaluate the role, if any, of *Acinetobacter* spp. and *P. aeruginosa* in MS.

ACKNOWLEDGMENTS

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Sub-acute, transmissible spongiform encephalopathies: current concepts and future needs

R. BRADLEY * and D. MATTHEWS **

Summary: The first diagnosis of bovine spongiform encephalopathy (BSE) in the United Kingdom in 1986 was to stimulate the most intensive epidemiological study of any animal disease of all time in that country. It led also to the initiation of a broad-based research programme with an international flavour. This principally involved scientists and veterinarians in Europe (especially the United Kingdom) and the United States of America, especially those with experience of slow infections in general and experimental scrapie in particular.

This final chapter highlights some of the significant discoveries made in the study of BSE and related diseases of this group but also emphasises the deficits in knowledge which need to be corrected before such diseases as scrapie in sheep and goats can be brought under control. The benefits resultant upon effective disease control will be manifest as improvement in animal production, welfare and, importantly, the removal of trading barriers currently in place to protect countries in which diseases such as BSE and scrapie do not exist.

Of key importance is the development of a simple, cheap and effective diagnostic test for use in the live animal before the onset of clinical signs. This will be difficult since the nature of the causal agents is uncertain and none provokes either a detectable immune response or inflammatory reaction in the host.

The earlier chapters, written by acknowledged specialists from around the world, deal with the specific diseases in detail and all present some of the most recent knowledge available. Here the authors emphasise the important role that major national and international agencies have in effecting the highest level of control possible in the absence of key information. International collaboration with countries in which these diseases exist, and as well as those where they are absent, is of paramount importance. It is essential that the BSE epidemic which has severely affected the cattle industry of the United Kingdom is not allowed to happen in developing countries. Whereas the former has implemented stringent control measures based on scientific knowledge and is well on the way to eradicating the disease, the latter could have much greater difficulty in establishing control. The answer is clear. BSE must be prevented from occurring elsewhere. To do that, knowledge of BSE and other members of the group should be widely dispersed and it is the purpose of this issue to do just that.

KEYWORDS: Bovine spongiform encephalopathy - Chronic wasting disease - Control - Nervous disease - Prevention - Prion disease - PrP - Scrapie - Sip gene - Transmissible mink encephalopathy - Transmissible spongiform encephalopathy - Virino.

* Ministry of Agriculture, Fisheries and Food, Central Veterinary Laboratory, New Haw, Addlestone, Surrey KT15 3NB, United Kingdom.

** Ministry of Agriculture, Fisheries and Food, Government Buildings (Toby Jug Site), Hook Rise South, Tolworth, Surbiton, Surrey KT6 7NF, United Kingdom.

CONTENTS

INTRODUCTION

For the 60th General Session of the OIE in May 1992, bovine spongiform encephalopathy (BSE) was selected as a major topic, particularly in view of the discussion on the *International Animal Health Code* Chapter on BSE. The *Code* Chapter is designed to provide additional information to facilitate trade in cattle and cattle products whilst minimising risk to importing countries.

Preceding papers in this *Review* deal with the specific "naturally-occurring" transmissible spongiform encephalopathies of animals. They consider the agents which cause them, and the factors controlling the occurrence of disease in an exemplary way, bringing together in a single journal much scientific information that is otherwise widely dispersed in scientific literature.

The purpose of this paper is to link these presentations, to contrast the animal diseases with their human counterparts, and to provide the most up-to-date field and research data, particularly with respect to BSE and the most recently discovered spongiform encephalopathies (SE) in other species. It also highlights deficits in knowledge which currently restrain governments, breed societies and farmers from implementing control measures (particularly in regard to scrapie) which are costly, usually draconian, and uncertain in outcome.

CONTRASTS BETWEEN ANIMAL AND HUMAN TRANSMISSIBLE SPONGIFORM ENCEPHALOPATHIES

The three human TSEs, Creutzfeldt-Jakob disease (CJD), Gerstmann-Sträussler (Scheinker) syndrome (GSS) and kuru are rare and have only been recognised and defined in the twentieth century. On the other hand, scrapie of sheep has been known and lucidly recorded in European literature since at least the early 1700s (13). All the other transmissible spongiform encephalopathies (TSEs) of animals have only been recognised since 1950.

Although all these diseases, human and animal, are infectious and have been transmitted experimentally, only scrapie of sheep (and perhaps goats and moufflon) is naturally contagious. Even in sheep, the only certain vehicle of contagion is the placenta and even that has been proven only from Swaledale sheep (17). Maternal transmission seems to be important in sheep scrapie, which is the reason why the disease is endemic and difficult to eradicate, but it does not occur, or is at least unproven, in all the other diseases.

Clearly in sheep, probably in goats and moufflon, and perhaps in all ruminants, it is important to reduce risks of transmission between animals by isolating animals at the time of parturition. Controlled destruction of placenta and foetal fluids, and cleaning and disinfection of the premises used for parturition are also appropriate before use by other animals. Only in sheep has it been shown that offspring from affected dams are likely to be infected, thus necessitating the removal of family lines if disease is to be controlled. It is, however, not inevitable that maternal transmission will occur in all offspring of affected dams. At least one gene of sheep (*Sip*), and

perhaps others, influence the length of incubation, neuroinvasion and occurrence of clinical disease. Consequently, identification of resistant animals may become an increasingly important feature of control.

The human disease, CJD, exists in three forms – familial (less than 15% of total cases) (14), sporadic (greater than 85%), and iatrogenic (extremely rare). GSS is also a familial disease and, like familial CJD, is associated with the occurrence of mutations in the *PrP* gene, both of which are hereditary. No such counterpart has been shown to occur naturally in animals but, if present, would appear to be at such a low incidence as to be undetectable. However, since such occurrence would probably result only from mutation, no country can be complacent that no animal SE (including BSE or scrapie) could ever occur in indigenous animals. Risks from such a development would be very small, but not absent.

The development of transgenic rodents with mutant *PrP* genes responsible for hereditary TSEs in humans is likely to be a very valuable research tool for further studies. The familial human spongiform encephalopathies are also transmissible, as are cases of the sporadic and iatrogenic forms of CJD (Ridley, personal communication).

Iatrogenic transmission of CJD is rare, but has resulted from the use of biological medicinal products (cornea, dura mater, human pituitary-derived growth hormone, human gonadotrophin) or insufficiently decontaminated neurosurgical instruments and depth EEG electrodes (22).

In animals, the most notable report of iatrogenic disease involved over 300 cases of scrapie caused by the use of a formalised louping ill vaccine prepared from sheep brains (11).

Iatrogenic transmissions demonstrate the need for safe sourcing of biological products since scrapie-like agents are extremely resistant to procedures that are totally effective in destroying conventional micro-organisms.

While the origin of the majority of CJD cases (sporadic CJD) is unknown, some suggest somatic mutation in the *PrP* gene to be the cause. In common with iatrogenic CJD however, sporadic CJD does not appear to be associated with diseases of animals, contact with animals, or use, or consumption of animal products. In other words, the animal diseases are not zoonoses as determined by all the evidence accrued from extensive epidemiological studies of the human diseases (4, 5, 6, 14, 22, 23).

The advent of BSE in 1986 rekindled quiescent thoughts on the risks to humans from the animal diseases and BSE in particular. The United Kingdom Department of Health has therefore funded a national surveillance programme for CJD commencing in May 1990, to identify any change in the pattern of CJD. This complements earlier studies by Professor W.B. Matthews (23) in England and Wales between 1970 and 1984. A retrospective survey to cover the period 1985 to 1990 is also in progress. Although the study has not yet been completed, the number of certified cases of CJD does not appear to have increased between 1980 and 1990. Parallel studies are being planned in other European countries, including the Netherlands and France, under the auspices of the Public Health Section of the European Community (EC) Scientific Veterinary Committee. The conclusion from all these studies is that as Professor Sir Richard Southwood reported the risk to man from BSE is remote (8).

CURRENT DATA ON CONFIRMED INCIDENTS OF SPONGIFORM ENCEPHALOPATHY IN ANIMALS

Cattle – bovine spongif rm encephalopathy

The first case of BSE was confirmed in November 1986 in England. Retrospective analysis of clinical histories has however suggested that the earliest case occurred around April 1985. Readers are reminded that Figure 1 of the paper by Kimberlin (page 354) includes such "early cases" where histopathological confirmation did not take place.

Lesions of spongiform encephalopathy have been demonstrated by brain histopathology in most confirmed cases of BSE. For small numbers of animals where autolysis has precluded such examination, diagnosis has relied on identification of scrapie-associated fibrils (SAF) in extracts of central nervous tissue. On 16 April 1992, 53,595 cases of BSE had been confirmed on 17,401 British farms. The youngest case was 22 months old and the oldest 17 years. The confirmed dairy herd incidence was 33%, the confirmed beef suckler herd incidence 4.7%, and the confirmed total herd incidence 18.2%. In other words, almost 82% of cattle herds in Great Britain had not experienced BSE.

BSE has been confirmed in several other countries listed by Kimberlin is this issue (12). With a constantly moving picture, readers are referred to individual State Veterinary Authorities for accurate statistics on their confirmed cases.

Sheep, goats and moufflon – scrapie

Presentation of current data on the prevalence and incidence of scrapie in various species in different countries is extraordinarily difficult. This is because the disease is only notifiable in some countries, generally those in which the disease is absent or has been eradicated.

Member Countries of the OIE report their scrapie status annually, and Table I summarises returns to April 1992 for the years 1980, 1985, 1990 and 1991. Table II summarises those countries in which scrapie has never been recorded, or the date of the last confirmed case. In other words, this lists countries which currently claim freedom from the disease.

As indicated in the paper by L.A. Detwiler (9), such statements need careful interpretation. They rely on the awareness of animal keepers of the clinical signs of scrapie and the importance of reporting them, as well as on the surveillance procedures in place to detect and confirm disease. This includes histopathological, electron microscopical (for SAF detection), and immunoblotting procedures to detect *PrP*^{Sc}. Furthermore, it is also necessary to have strict control of importation, compulsory notification, and a planned stamping-out policy should disease ever be detected.

The OIE publication *Animal Health in the Eighties* (15), summarises scrapie control procedures in place in certain Member Countries, namely: compulsory notification, stamping-out, modified stamping-out and prohibition of imports from infected countries. These are reproduced in Table III.

TABLE I

Summary of the scrapie status in countries which reported to the OIE
(1980, 1985, 1990 and 1991)

Countries	1980	1985	1990	1991
Afghanistan	-	-	...	...
Albania	...	-	0000	
Algeria	-	-	...	...
Angola	-	0000	...	...
Antigua and Barbuda	-	-	-	
Argentina	-	0000	0000	0000
Australia	-	-	(1952)	(1952)
Austria	-	-	-	-
Bahamas	-	0000	0000	
Bahrain		-	+	...
Bangladesh	...	...	...	...
Barbados	(-)	-	0000	
Belgium	(-)	0000	-	-
Belize	-	-	0000	
Benin	...	...	0000	
Bhutan	(-)	-	-	-
Bolivia	0000	0000	0000	0000
Botswana	0000	0000	0000	0000
Brazil	-	+⇐	-	(1985)
Bulgaria	-	...	0000	0000
Burkina Faso	-	...	-	-
Burundi	...	-	0000	
Cameroon	...	...	-	
Canada	(+)	+	(+)	(+)
Cape Verde	...	...	...	
Cambodia	-	-	-	
Central African Republic	-	?	...	
Chad	?	...	...	
Chile	-	0000	0000	0000
China (People's Rep. of)	...	...	...	
Colombia	(-)	-	(1976)	(1976)
Congo	(-)	-	-	-

TABLE I (contd)

Summary of the scrapie status in countries which reported to the OIE
(1980, 1985, 1990 and 1991)

Countries	1980	1985	1990	1991
Costa Rica	-	0000	0000	
Cote d'Ivoire	...	...	-	-
Cuba	-	-	0000	0000
Cyprus	-	+!	+()	+()
Czech and Slovak Fed. Rep.	-	-	-	(+)
Denmark	0000	0000	0000	0000
Djibouti	-	0000	...	...
Dominican Rep.	0000	0000	0000	
Ecuador	0000	0000	0000	0000
Egypt	-	0000	0000	0000
El Salvador	-	0000	0000	0000
Ethiopia	-	0000	0000	
Falkland Islands	-	-	-	
Finland	-	0000	0000	0000
France	+()	+	+	+
Gabon	-	-	-	
Germany	-	0000	0000	
Ghana	-	0000	0000	0000
Greece	0000	0000	0000	0000
Guatemala	0000	0000	0000	
Guinea	-	-	...	
Haiti	-	-	-	
Honduras	-	-	0000	
Hong Kong	0000	0000	0000	
Hungary	(-)	0000	0000	0000
Iceland	+()	+()	(+)	
India	?	0000	0000	0000
Indonesia	-	-	...	...
Iran	-	0000	0000	
Iraq	-	...	0000	
Ireland	+	+	+	+

TABLE I (contd)

Summary of the scrapie status in countries which reported to the OIE

(1980, 1985, 1990 and 1991)

Countries	1980	1985	1990	1991
Israel	0000	0000	0000	0000
Italy	(+)	...	...	...
Japan	-	+	+	+
Jordan	-	...	...	...
Kenya	-	0000	...	...
Korea (Rep. of)	-	-	0000	0000
Kuwait	-	-	-	
Laos	...	...	...	...
Lebanon	(+)	-	-	-
Lesotho	0000	0000	0000	
Libya	-	-	0000	
Luxemburg	-	0000	0000	0000
Madagascar	-	0000	0000	
Malawi	-	0000	0000	0000
Malaysia (peninsular)	-	0000	0000	
Malaysia (Sabah)	-	-	-	-
Malaysia (Sarawak)	-	-	-	
Mali	-	-	...	-
Malta	-	-	-	-
Mauritania	-	0000	-	-
Mauritius	-	-	0000	
Mexico	-	0000	0000	0000
Mongolia	...	...	0000	
Morocco	-	0000	0000	0000
Mozambique	-	-	-	-
Myanmar	+	-	...	0000
Namibia	0000	0000	0000	0000
Nepal	-	-	+	
Netherlands	(+)	+	?	?
New Caledonia	(-)	0000	0000	0000
New Zealand	(1954)	(1954)	(1954)	(1954)

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TABLE I (contd)

Summary of the scrapie status in countries which reported to the OIE

(1980, 1985, 1990 and 1991)

Countries	1980	1985	1990	1991
Niger	...	0000	0000	
Nigeria	...	0000	0000	0000
Norway	(1956)	+	-	(+)
Oman	...	0000	0000	
Pakistan	-	...	...	0000
Panama	-	0000	0000	0000
Paraguay	-	0000	0000	
Peru	0000	0000	...	
Philippines	-	-	0000	
Poland	-	0000	0000	0000
Portugal	-	-	0000	
Romania	-	0000	0000	0000
Saint Kitts and Nevis	...	...	0000	
Saint Lucia	-	-	-	
Saudi Arabia	-	...	...	...
Senegal	-	+	...	...
Seychelles	-	-	-	
Sierra Leone	-	...	...	
Singapore		-	0000	0000
Somalia	-	-	-	
South Africa	(1972)	(1972)	(1972)	(1972)
Spain	0000	0000	-	...
Sri Lanka	-	-	0000	0000
Sudan	...	-	-	0000
Swaziland	0000	-	...	...
Sweden	-	0000	(1986)	(1986)
Switzerland	-	?	-	(+)
Syria	?	0000	0000	-
Taiwan R.O.C.	...	0000	0000	0000
Tanzania	-	-	...	
Thailand	-	0000	0000	0000

TABLE I (contd)

Summary of the scrapie status in c untries which reported to the OIE
(1980, 1985, 1990 and 1991)

Countries	1980	1985	1990	1991
Togo	(-)	0000	-	
Trinidad and Tobago	-	-	0000	
Tunisia	-	-	-	-
Turkey	-	+	-	
Uganda	?	-	0000	0000
United Arab Emirates	-	0000	...	0000
United Kingdom (G.B.)	+	+	+	+
United Kingdom (N. Ireland)	(1972)	(1972)	(+)	(+)
United States of America	+	+	+	+
Uruguay	0000	0000	0000	0000
USSR	0000	0000	0000	
Vanuatu	-	0000	0000	0000
Venezuela	-	-	-	-
Vietnam	-	-	0000	
Virgin Islands	...	...	0000	
Yugoslavia	-	+	...	...
Zaire	-	+	...	
Zambia	0000	0000	0000	0000
Zimbabwe	-	0000	0000	0000

CODE

Disease occurrence

0000 Never reported
\- Not reported
(-) Not recorded; probably not present
(Year) Year of last occurrence
? Suspected but not confirmed
(+) Exceptional occurrence
\+ Disease present
() Confined to certain regions
! Recognised in country for the first time
⇦ Only in imported animals (quarantine)
... No information available

TABLE II

Countries which claim freedom from scrapie
(reports received in 1990 and 1991)

C untries reporting freedom from scrapie	
Albania	Malawi
Argentina	Malaysia (Peninsular)
Australia: 1952	Mauritius
Bahamas	Mexico
Barbados	Mongolia
Belize	Morocco
Benin	Myanmar
Bolivia	Namibia
Botswana	New Caledonia
Brazil: 1985	New Zealand: 1954
Bulgaria	Niger
Burundi	Nigeria
Chile	Oman
Colombia: 1976	Pakistan
Costa Rica	Panama
Cuba	Paraguay
Denmark	Philippines
Dominican Republic	Poland
Ecuador	Portugal
Egypt	Romania
El Salvador	St Kitts and Nevis
Ethiopia	Singapore
Finland	South Africa: 1972
Germany	Sri Lanka
Ghana	Sudan
Greece	Sweden: 1986
Guatemala	Syria
Honduras	Taiwan R.O.C.
Hong Kong	Thailand
Hungary	Trinidad and Tobago
India	Uganda
Iran	United Arab Emirates
Iraq	Uruguay
Israel	USSR
Korea (Rep. of)	Vanuatu
Lesotho	Vietnam
Libya	Virgin Islands
Luxembourg	Zambia
Madagascar	Zimbabwe

Year: date of the last confirmation of disease

Source: OIE *World Animal Health*, Vol. VI, 1990 and Reports received for publication in *World Animal Health*, Vol. VII, 1991

TABLE III

Summary of policies of certain countries in regard to scrapie control

Countries	Control policy			
	CN	SOP	MSOP	POI
Argentina	+			+
Australia	+			
Botswana				+
Brazil				+
Cuba	+			
Cyprus	+		+	+
Denmark				+
Ecuador				+
El Salvador	+			
Finland	+	+		+
Germany				+
Israel	+			
Korea (Rep. of)				+
Mexico	+			+
New Caledonia				+
New Zealand	+			+
Norway	+	+		+
Philippines				+
Romania				+
South Africa	+	+		
Sweden	+	+		+
Trinidad and Tobago				+
Turkey	+			
USA	+		+	
USSR	+			
Vanuatu				+
Venezuela	+			+

CN = compulsory notification
SOP = stamping-out policy
MSOP = modified stamping-out policy
POI = prohibition on imports from infected countries

Source: Animal Health in the Eighties (15)

Cats – feline spongiform encephalopathy

The first case of feline spongiform encephalopathy (FSE) in a domestic cat was recognised in April 1990 (26). To date, 26 cases have been confirmed, one of which was in Northern Ireland and another on the island of Guernsey. The source of infection is unknown but is presumed to be food. Cat to cat transmission has not been reported, but, experimentally, the disease has been transmitted to mice by parenteral inoculation of cat brain tissue. Transmission was successful with both unfixed brain and tissue previously fixed in formalin.

Spongiform encephalopathy has recently been diagnosed in other carnivores (captive cheetah and puma) (16, 25).

Exotic Bovidae – spongiform encephalopathy

A total of 10 cases have been identififed in five species of exotic Bovidae captive in British zoos or wildlife parks. These species are nyala (*Tragelaphus angasi*: 1 case), gemsbok (*Oryx gazella*: 1 case), Arabian oryx (*Oryx leucoryx*: 1 case), greater kudu (*Tragelaphus strepsiceros*: 3 cases) and eland (*Taurotragus oryx*: 4 cases). One of the kudu was the offspring of a confirmed case and may have been the consequence of maternal transmission.

Transmission to mice from fixed brain tissue of the nyala and kudu has been successful, confirming that disease in these individuals is a TSE. Apart from the kudu offspring case, most of the other individuals had access to food or pasture potentially contaminated with infected offals or protein derived from them.

It is important to note the close relationship between these exotic ruminants and cattle, sheep and goats, which all fall within the family Bovidae. They are however quite distinct from deer, which belong to the family Cervidae.

Deer – chronic wasting disease

Chronic wasting disease (CWD) is confined to mule deer (*Odocoileus hemionus hemionus*), black-tailed deer (*O. hemionus columbianus*), mule deer crossed with white-tailed deer (*O. virginianus*) and Rocky Mountain elk (*Cervus elaphus nelsoni*) in Colorado and Wyoming in the United States of America (24). No such disease has been identified anywhere else in the world, with the exception of one case in a Canadian zoo.

Concern has been expressed about another disease of deer, "Chronic ill thrift", which occurs in New Zealand, but this has no connection either aetiologically or pathologically with CWD in particular or SEs in general. There is no specific cause, but a scrapie-like disease can be definitely excluded as a result of rigorous investigations carried out by the New Zealand veterinary authorities.

Pigs, chickens and other domesticated livestock reared for food

No naturally-occurring spongiform encephalopathy has been identified in these species anywhere in the world. In the United Kingdom, and some other countries, veterinary surveillance schemes in operation are extensive and any significant incidence of a progressive neurological disease would be detected by clinical examination supported by histopathological examination of brains.

Mink – transmissible mink encephalopathy

Transmissible mink encephalopathy (TME) has never been diagnosed in mink in the United Kingdom or indeed any countries in which BSE has been confirmed.

Ostriches – spongiform encephalopathy

Ostriches kept in German zoos showed lesions of spongiform encephalopathy (18, 19), but transmissibility and the presence of SAF and abnormal *PrP* have still to be demonstrated. Therefore, doubt still prevails in regard to the cause of this disease.

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CERTIFICATION AND TRADE

The appearance of BSE has emphasised the need for rigorous certification of imported animals and some animal products in species affected by spongiform encephalopathies. Since there is no practical effective test to detect the presence of disease in the live animal, all certification must be circumspect and carry an element of risk. Nevertheless, it is possible to reduce this risk to a minimum, as detailed in the chapters in this *Review* on BSE and scrapie (9, 12).

Clearly, with different routes of transmission, BSE and scrapie require the application of different strategies for control and certification. In BSE, where the only significant route of infection is considered to be via feed, it is relatively simple to certify country and animal freedom at an appropriate time, particularly if a food source is eliminated, and if assurances can be given that animals for export are not the offspring of affected cases. Provided that appropriate mandatory controls, such as a ban on feeding ruminant protein to ruminant animals, are in place and adhered to, the risk factors are minimal, even from countries with significant numbers of confirmed cases.

With scrapie, a different strategy must be adopted, and applied, at the flock level. This is because it is a flock disease, unlike BSE, with both maternal and horizontal transmission playing a role in perpetuating disease. Furthermore, the *Sip* gene exerts an important control over disease occurrence and may mask the presence of infection. Thus it is only possible to certify disease freedom when all animals are clearly and permanently identified, breeding records are meticulous, audited by a competent authority, and thorough pathological investigation of neurological disease is implemented. To satisfy definition of country freedom from scrapie would mean that every flock within the country would have to satisfy the criteria specified. Since the majority are entirely commercial non-pedigree flocks, this is an unnecessary burden for countries and individual farmers to carry.

It is however important that flocks within countries which export breeding stock abide by strict rules in order to reduce the risk of scrapie transmission to an acceptable minimum.

In the EC, scrapie must become a notifiable disease by 1 January 1993. The Community is currently considering how such certification can be implemented in order to foster internal trade in live animals for breeding whilst strictly controlling the risk of transmitting disease. This will be done by adopting rules on a flock basis similar to those in place in the United Kingdom Sheep and Goat Health Scheme scrapie option (9). Inevitably, the validity of certification of freedom from disease will always be enhanced the longer freedom can be maintained. With a modal incubation period for natural scrapie of about three and a half years, freedom for less than this period is meaningless. Five years' freedom might give a small degree of assurance, but while confidence does progressively increase with time, absolute guarantees may prove impossible given the genetic influences on disease expression.

Governments and individuals should therefore be aware of the risks of introducing disease, and of ways of reducing them to acceptable levels, whilst also permitting genetic improvements through the importation of animals, semen and embryos of superior genetic merit. Embryo transfer is, as yet, not proven to be a completely safe procedure in eliminating scrapie, but this subject will be discussed further below.

A major problem remains how to enable countries now generally regarded as free from scrapie to become integrated without penalty into a new international trading scheme. This may become the subject for future discussion, but the lack of accurate data on disease incidence world-wide means that assessment of risk before trading commences may be difficult.

RESULTS OF RESEARCH STUDIES ON TRANSMISSION FROM TISSUES OF BSE-AFFECTED CATTLE

Host range studies

BSE has been successfully transmitted directly to scrapie-susceptible strains of mice (now an experimental model system), cattle, sheep, goats, pigs and marmosets, but not to hamsters (this required prior sub-passage via mice). The diseases and the neuropathology so produced in cattle, sheep and goats are broadly similar to the natural diseases of those species. The results of attempted transmission to mink are pending.

The transmission to sheep, goats and mice is not unexpected, that to pigs of little consequence, other than to stimulate the extension in the United Kingdom of the prohibition on the use of specified bovine offals for food to all species. The transmission to marmosets may concern some, but it should be recalled that scrapie was transmitted many years ago to primates (but not to chimpanzees). The minimum incubation period in marmosets inoculated with scrapie was, at 38 months, substantially greater than that in experiments involving CJD (less than 22 months). BSE took even longer (48 months). These differences may however merely reflect agent titre in the inoculum. It would appear that the species barrier between cattle and marmosets is nevertheless substantial, although not absolute. It should be remembered that the experiment was initiated before successful transmission to mice was achieved, and might have provided very valuable information had transmission occurred more rapidly and had no transmission occurred in the other experimentally-challenged species. Now the result is more of comparative historical interest, but serves to show that transmission risks are measurable and steps need to be taken (and have been) to protect wild and other species from exposure to the BSE agent.

Tissue transmission studies

A wide range of tissues from BSE-affected cattle have been inoculated parenterally into susceptible mice. So far, transmission has only succeeded with brain tissue. Some experiments have been in progress more that 700 days from inoculation, which is beyond the accepted scientific end-point for a negative transmission of SEs. The mice remain clinically healthy. The tissues involved (days post-inoculation are given in parentheses) are, at 23 April 1992, spleen (994); semen (967); buffy coat of blood (904); muscle [*M. diaphragma*] (896); muscle [*M. semitendinosus*] (899); placenta (811); and bone marrow (780).

Oral challenge studies of mice with various tissues are now complete, and only brain/cerebrospinal fluid resulted in transmission. There was no detectable infectivity after massive challenge with spleen, mesenteric and carcass lymph nodes,

supramammary lymph nodes, udder/milk, or placenta (1, 2) even after now completed blind parenteral passage of spleen and spinal cord at 12 and 18 months post-inoculation (Barlow, personal communication).

These studies collectively show that there is no detectable infectivity in the listed tissues, as revealed by the assay system used. Thus BSE differs significantly from scrapie with respect to tissue distribution of agent. Of particular importance is the absence of detectable infectivity in semen or placenta, the former in line with findings for scrapie using a mouse model. Oronasal exposure of cattle to placenta is in progress (challenged at the end of 1989), but no clinical disease has resulted to date.

Offspring and embryo transfer studies in cattle are also in progress. In the former, over 300 pairs of cattle (pairs means an offspring of a confirmed case plus a control from the same farm with a live, aged dam) are being held on Ministry of Agriculture farms for observation (without mating).

The embryo transfer study involves transfer of embryos from field cases of BSE, mated by artificial insemination to either healthy or BSE-affected bulls. Embryos are transferred according to the International Embryo Transfer Society protocols, and recipients, imported from New Zealand, are kept in quarantine on a Ministry of Agriculture farm. To date, over 100 of a total of 350 eventual recipients have confirmed pregnancies. The first few calves have been born. Offspring and recipients will be observed until they are seven years of age before slaughter and examination of brains for spongiform encephalopathy. Any that die during the course of the experiment will of course be subjected to the same examination.

The initial results have now been published of an experiment which forms part of a series of studies involving embryo transfer from sheep inoculated experimentally with scrapie (10). In contrast to the studies in cattle, donor and recipient Cheviot sheep were selected by their *Sip* genotype, determined by restriction fragment length polymorphism (RFLP) analysis of the *PrP* gene. Donor ewes were inseminated with semen from a healthy unexposed ram, and embryos were transferred without washing in order to enhance the possibility of transmission. Recipients (*Sip* pApA but including one sApA) were not susceptible to natural scrapie. Offspring were of three genotypes (sAsA, sApA, pApA). Six *Sip* sAsA sheep developed scrapie at approximately two years of age, but the others (11 sApA and 3 pApA) were still alive at the time of publication.

This experiment demonstrates that the embryo, under the conditions of the experiment, may transmit infection, but whether on the surface of the zona pellucida or within the substance of the embryo is unknown. This is the subject of further studies. This experiment does not call into question the safety of bovine embryos with respect to transmission of BSE, but guidelines to prevent transmission, either of scrapie or BSE via embryos, have been proposed within EC and OIE Working Groups. Briefly, in regard to scrapie, it is recommended that movement of embryos or semen should be restricted to that between flocks of equivalent scrapie status, or from higher to lower status flocks. In regard to BSE, there are no proposals for restrictions on the use of semen, but embryos should not be derived from animals that have consumed ruminant protein, have been affected with BSE or from their daughters.

BSE IN ANIMALS BORN AFTER THE INTRODUCTION OF THE RUMINANT FEED BAN IN JULY 1988

There have been some 38 cases confirmed in such animals so far. While to the casual observer this might suggest an origin of infection other than food, nothing could be further from the truth. Only in one instance does it appear that maternal transmission is likely. The dam of this case was BSE-positive, and a food source was effectively eliminated. While some investigations are still in progress, it is considered that most of these cases were at some risk of exposure via food, largely through carry over of stocks manufactured before the feed ban, or due to accidental inclusion of meat-and-bone meal.

It should be stressed however that the confirmed cases in this age group are far fewer than expected if there had been no ruminant feed ban. In other words, while the cut-off point for cessation of feeding of ruminant protein was not as precise as intended, it has had an effect on disease incidence (21). For example, the incidence in two-year-old cattle (over two but less than three years of age) was lower in 1991 (0.01%) than in 1990 (0.05%). Further analyses in progress support this encouraging picture. With each passing calving year, the ban should progressively reduce the incidence in older age groups, eventually significantly affecting the overall report rate four to five years after the introduction of the feeding ban (the modal incubation period being four and a half years) – in effect, through the winter calving season of 1992-1993. Nevertheless, while such data are encouraging, because only relatively small numbers of affected cattle at the younger end of the age range are currently involved, the authors caution against drawing firm conclusions at this stage.

OTHER ENIGMAS

At every turn, research into this group of diseases produces unexpected findings. The nature and molecular biology of the agents admirably described in the chapter by Bennett and colleagues is a case in point (3). At the more practical level, a short letter to the *Veterinary Record* by Clark (7), a veterinary investigation officer with wide experience of scrapie, describes a study involving 192 submissions, where 15% of suspect cases of scrapie exhibiting classical signs of pruritus, emaciation, ataxia and hyperaesthesia gave negative diagnoses on histopathological examination of brain tissue. In the same study, 21% of sheep found dead for no obvious reason were histopathologically positive for scrapie. Clearly such observations are important and should be accommodated when drafting protocols for scrapie control or certification.

METHODS AND CRITERIA FOR BSE DIAGNOSIS

At an early stage in the epidemic it was recognised that diagnostic methods for BSE should be harmonised both nationally and internationally. Two diagnosis workshops (1990 and 1991) have been held for veterinary neuropathologists from

other countries under the auspices of the EC at the Central Veterinary Laboratory, Weybridge. The histological procedures used for BSE diagnosis in the United Kingdom are detailed in Appendix 1.

CONCLUDING REMARKS

It has been a fascinating and enjoyable task editing, albeit at some speed, this volume of the *Review*. No doubt some errors will have escaped our attention and for that we apologise. Nevertheless, the quality of the other chapters will surely atone for any editorial defects. What is clear from the papers presented here is that in a comparatively short space of time, an enormous amount of valuable information has been collected. This has formed the basis of control procedures admirably supported by the EC, OIE, FAO, WHO and other national and international bodies. It is now up to Governments world-wide to make realistic assessments of risks, at home and abroad, while encouraging minimal risk trading in ruminant animals and their products (relative to TSEs). All trading countries can be expected in the future to have to provide sound data in support of claims of freedom from specific diseases of this group, at least until practical and effective diagnostic tests and surveillance procedures are developed for use in live animals of all ages.

Further work is needed to determine the role, if any, of sheep of *Sip* pApA genotype in transmitting infection. More studies are required to determine:

- how scrapie is transmitted between sheep;
- the vehicle of transmission; and
- the importance of environmental contamination.

Guidance is also needed on effective decontamination methods for scrapie-like agents to apply to premises, animal products (e.g. rendered waste for animal feed), and surgical equipment. Indeed, such studies are already in progress. Knowledge is now available on the *Sip* gene and there are practical methods to detect genotype by RFLP analysis. This technology should be exploited in flocks naturally infected with scrapie and in which challenge can be maintained at the previous level, thereby determining the effectiveness of breeding for "resistance" (long incubation) in extreme conditions of challenge. This will involve collaboration between farmers, veterinarians, researchers and administrators. The rewards could be enormous, to the benefit of both animal health and world-wide trade.

Finally, vigilance must be maintained in our fight against these diseases, particularly in the area of identification of safe sources of biological products and devices. Current controls on all aspects of BSE seem at least adequate as practised in the United Kingdom. Controls beyond this are unjustified and it is hoped that the discussions and conclusions of the General Session and the data in the papers in this volume will aid those less familiar with the diseases to reach sensible decisions on future actions in regard to trade.

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ENCÉPHALOPATHIES SPONGIFORMES SUBAIGUËS TRANSMISSIBLES : CONCEPTS ACTUELS ET BESOINS FUTURS. – R. Bradley et D. Matthews.

Résumé: Le premier cas d'encéphalopathie spongiforme bovine (BSE) diagnostiqué au Royaume-Uni en 1986 fut à l'origine des études épidémiologiques les plus poussées qui aient jamais été réalisées sur une maladie animale dans ce pays. Il s'en est également suivi la mise en place d'un vaste programme de recherche, avec des prolongements internationaux. Ce sont essentiellement des scientifiques et des vétérinaires spécialisés dans les infections à virus lents et dans la tremblante expérimentale qui ont participé aux travaux en Europe (surtout au Royaume-Uni) et aux Etats-Unis.

Ce chapitre final est consacré aux découvertes importantes réalisées sur la BSE et les maladies apparentées et met en lumière les questions qui restent à élucider pour maîtriser des affections comme la tremblante des ovins et caprins. Les avantages d'une prophylaxie efficace se traduiront par des améliorations de la production et de la santé animales et surtout par l'élimination des barrières commerciales qui ont été mises en place pour protéger les pays indemnes des maladies du type de la BSE et de la tremblante.

La mise au point d'une épreuve diagnostique simple, peu coûteuse et efficace, utilisable chez l'animal vivant avant l'apparition des signes cliniques, est d'une importance capitale. Cette étape sera pourtant difficile car la nature des agents pathogènes est incertaine et aucun d'eux n'entraîne de réponse immunitaire décelable ou de réaction inflammatoire.

Les chapitres antérieurs, écrits par des spécialistes renommés du monde entier, présentent les spécificités de chaque maladie ainsi que les résultats des recherches les plus récentes. A cet égard, les auteurs soulignent le rôle des principaux organismes nationaux et internationaux dans la mise en œuvre de la meilleure prophylaxie possible, malgré l'absence de certaines informations clés.

La collaboration internationale entre les pays touchés par ces maladies et les pays indemnes est indispensable. Il est essentiel que l'épidémie de BSE qui a sévèrement touché la production bovine au Royaume-Uni ne se propage pas aux pays en développement. En effet, si le Royaume-Uni a mis en œuvre des mesures de prophylaxie efficaces fondées sur des connaissances scientifiques et qu'il est aujourd'hui sur la voie de l'éradication, certains pays risqueraient

fort de rencontrer des difficultés majeures dans une telle démarche. La réponse ne fait aucun doute : il est impératif de prévenir l'apparition de la BSE dans toute autre partie du monde. A cette fin, il convient de diffuser largement les informations sur la maladie et les autres affections apparentées, et c'est précisément l'objet de cette publication.

MOTS-CLÉS : Cachexie chronique - Encéphalopathie spongiforme bovine - Encéphalopathie spongiforme transmissible - Encéphalopathie transmissible du vison - Gène Sip - Maladies à prion - Maladies du système nerveux - Prophylaxie - PrP - Tremblante - Virino.

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ENCEFALOPATÍAS ESPONGIFORMES TRANSMISIBLES SUBAGUDAS: CONCEPTOS ACTUALES Y NECESIDADES FUTURAS. – R. Bradley y D. Matthews.

Resumen: El primer diagnóstico de encefalopatía espongiforme bovina (BSE) en el Reino Unido, en 1986, fue un estímulo para el estudio epidemiológico más intensivo jamás realizado en ese país en relación con una enfermedad animal. Además, fue motivo para iniciar un amplio programa de investigación internacional en el que intervienen principalmente científicos y veterinarios de Europa (sobre todo del Reino Unido) y de los Estados Unidos de América, en especial aquellos que poseen experiencia en las infecciones por virus lentos en general y en el prurigo lumbar experimental en particular.

Este último capítulo pone de relieve algunos de los hallazgos más significativos realizados en el marco del estudio de la BSE y demás enfermedades de este grupo, subrayando así mismo las faltas de conocimientos que deben subsanarse antes de que las enfermedades como el prurigo lumbar de ovinos y caprinos puedan controlarse. Las ventajas que resultarán de ello para un control eficaz de las enfermedades son evidentes: mejora de la producción pecuaria, mayor bienestar de los animales y, lo que es aún más importante, eliminación de las barreras comerciales existentes actualmente para proteger a los países en que no existen la BSE y el prurigo lumbar.

El desarrollo de una prueba de diagnóstico sencilla, barata y eficaz para su empleo en animales vivos antes de que aparezcan los signos clínicos reviste una importancia decisiva. Sin embargo, esto será algo difícil de lograr, pues la naturaleza de los agentes causantes aún no se ha definido con certeza y ninguno de ellos provoca una respuesta inmunológica detectable ni una reacción inflamatoria en los huéspedes.

Los capítulos anteriores, escritos por reconocidos especialistas del mundo entero, tratan detalladamente de las enfermedades específicas y presentan los conocimientos disponibles más recientes. En el presente artículo, los autores subrayan el importante papel que desempeñan los principales organismos nacionales e internacionales en la ejecución del mayor control posible en ausencia de informaciones claves. La colaboración internacional entre los países afectados por estas enfermedades y aquellos que están libres de ellas es pues de suma importancia. De igual modo, es esencial que la epidemia de BSE que ha afectado gravemente la industria ganadera del Reino Unido no llegue a los países en desarrollo, ya que, mientras el primero ha aplicado medidas de control estrictas, basadas en el conocimiento científico, y está en camino de erradicar la enfermedad, los segundos tendrían enormes dificultades para lograr ese control. La respuesta es clara: debe evitarse que la BSE se propague a otras partes del

mundo y, con tal fin, los conocimientos acerca de la BSE y las otras enfermedades emparentadas deben difundirse ampliamente. Esta es precisamente la finalidad de la presente publicación.

PALABRAS CLAVE: Caquexia crónica - Encefalopatía espongiforme bovina - Encefal patía espongiforme transmisible - Encefalopatía transmisible de los visones - Enfermedades del sistema nervioso - Enfermedades causadas por un prion - Gen Sip - Prevención - PrP - Prurigo lumbar - Virino.

* * *

Appendix 1

:PTOS

PROTOCOLS FOR THE DIAGNOSIS OF BOVINE SPONGIFORM ENCEPHALOPATHY IN THE UNITED KINGDOM

Introduction

For countries where BSE does not exist, it is important that, if clinical suspects are notified or detected, appropriate samples of central nervous system tissue are collected and prepared in a manner that will permit diagnosis, differential diagnosis and additional studies to support a diagnosis of spongiform encephalopathy (SE) should it be made.

It is essential to maximise the fixed brain tissue collected for histopathology in order that a diagnosis of SE is not missed, the bilateral symmetry of lesions is checked and differential diagnoses are made or eliminated.

In the United Kingdom there is a remarkable consistency in the distribution of lesions between cases of BSE though the severity may vary. Lesions concentrate in the brain stem, thus permitting statutory diagnosis to be made by examination of a single section of the medulla oblongata at the obex. However it is possible that different strains of BSE agent may occur in different countries, and these could theoretically cause disease with a different lesion distribution which might be missed if only a single medulla section was examined in the early stages of an epidemic. Once a sufficient number of cases have occurred with a consistency of lesion distribution, a more reduced examination may be feasible and adequate. Thus the following describes the procedure for full brain removal (which should be followed in the early stages of an epidemic, together with collection of 5-10 g of frozen unfixed cervical spinal cord for fibril [SAF] and *PrP* examination by an appropriate laboratory). Also included is a description of a procedure for removal of the brain stem via the foramen magnum which is currently used in the United Kingdom and may be appropriate in other countries once confirmation of the consistency of lesion distribution in the appropriate part of the brain stem is made.

Tissue for SAF or *PrP* examination should be frozen pending delivery to an appropriate laboratory. The procedures for these studies are not included here. Studies in the United Kingdom have shown that SAF can be detected in positive cases of BSE despite autolytic changes in the brain. However, histopathology is the method of choice for diagnosis and is accepted internationally, including in the European Community.

Removal of the brain for histological examinati n

Disarticulate the head from the neck at the atlanto-occipital joint. Remove the skin from the dorsal surface of the head. The head should then be fixed firmly in a vice.

Cut laterally through the frontal bone just dorsal to the orbits with a butcher's saw. Further cuts are then made on each side through the frontal bone from points just axial to the orbits extending caudally to a point just dorsal of the external auditory meatus. These cuts are then extended caudally to the foramen magnum.

Remove the roof of the cranium by leverage using a bone wedge and bone forceps if necessary.

Dissect and remove the dura mater. The front of the brain should be lifted gently while a scalpel is used to cut the cranial nerves on the ventral surface. By working towards the spinal cord the whole brain can be removed from the skull without damage and can be carefully placed in a bucket containing ten times the brain volume (4-6 litres) of 10% formol saline:

Sodium chloride	8.5 g
Formalin (40% HCHO)	100 ml
Distilled water	900 ml

Dissolve sodium chloride in water, add formalin and mix.

Fixation

Whole brain is fixed in 10% formal saline for two weeks prior to cutting-in. Change the fixative after one week.

Slice the brain transversely at 3-5 mm intervals. Select four brain stem blocks for processing. Recommended sites are: medulla at obex, medulla through caudal cerebellar peduncles and midbrain at two levels to include the superior colliculus and the red nucleus. In countries where BSE has yet to be confirmed, a more complete neurohistological examination is advised.

The selected blocks are returned to 10% formol saline for a further week during which fixative should be changed on three occasions before processing. Mechanical agitation will aid penetration of the fixative.

THE FORAMEN MAGNUM TECHNIQUE

By careful dissection of the spinal nerves and meninges the required pieces of brain tissue containing the necessary target sites can be removed via the foramen magnum by using a wire loop and stainless steel tube. The tube should measure 200 mm × 5 mm and be made of stainless steel to permit disinfection; 0.8 mm mild steel wire is fed through the tube to make a loop at one end. The principle of the extraction technique is depicted in Figures 1a and 1b. The loop is passed up the brain stem to a position just forward of the pons and the wire loop drawn through to cut the brain at this point. The brain stem can then be removed in one piece by careful traction through the foramen magnum. An alternative to the wire loop method is to use a stainless steel spade-shaped cutting tool rigidly fixed to a handle at an angle. A separate spoon-shaped tool is useful to remove the cerebellum or this can be fixed to the other end of the cutting tool. Tools can be made in a local workshop.

Procedure

1. Place the head dorsal side down on a necropsy table.
2. By careful dissection, separate the medulla from the dura mater and sever the spinal nerves. (To facilitate this, a headlamp such as one used for caving, greatly facilitates the visualisation of the brain within the cranial cavity.)
3. Elevate the medulla to expose the cerebellar peduncles and sever using a conventional flat brain knife passed forward to the level of the midbrain.
4. Remove the severed cerebellum from the cranial cavity using the spoon-shaped tool.
5. a) Grasp the protruding spinal cord carefully using rat-tooth forceps and insert the wire loop over the medulla. Manipulate the wire over the severed cerebellar peduncles, and pass the tube up to the level of the pons. When the positioning requirements are satisfied, draw the wire tight to sever the brain stem.
5. b) Alternatively, insert the spade-shaped tool through the foramen magnum with the pointed end aimed dorsally to the level of the pons. Now rotate the tool to sever the brain stem.
6. Remove the brain stem by careful traction and place in approximately 500 ml of formol saline for primary fixation for one week.
7. After primary fixation, the required target sites are selected for secondary fixation for 24 hours, using an orbital shaker, then processed as for standard protocols.
8. All the wire loops and disposable instruments and materials should be incinerated. Instruments should be autoclaved in a porous load autoclave at 136°C-138°C for 18 minutes at 30 lbs psi hold temperature and time (HTAT). A suitable chemical disinfectant is sodium hypochlorite providing 2% of available chlorine allowed to act for 1 hour in the absence of organic matter. Alternatively 1M sodium hydroxide could be used similarly if corrosion presents a problem.

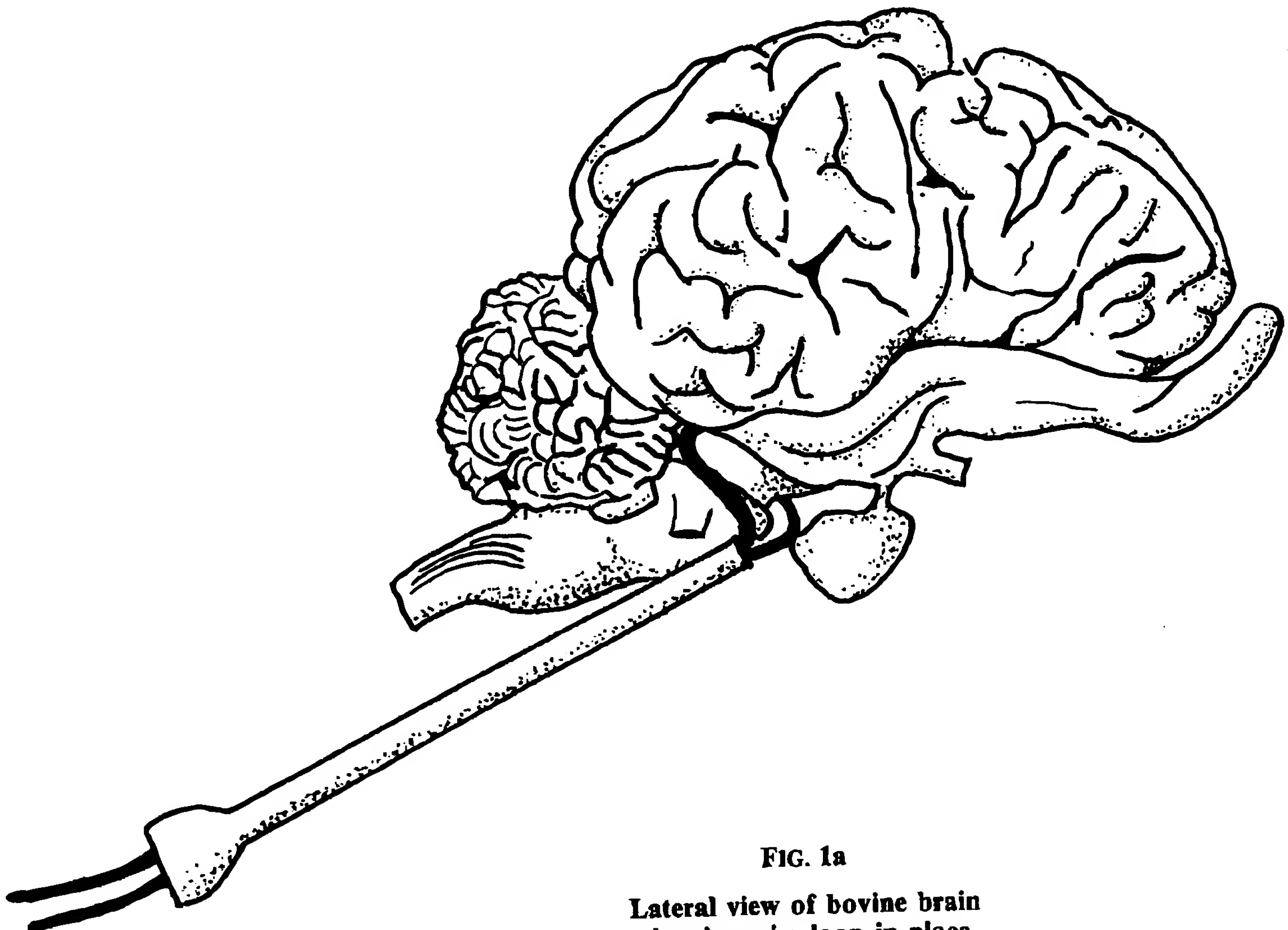

FIG. 1a

Lateral view of bovine brain showing wire loop in place

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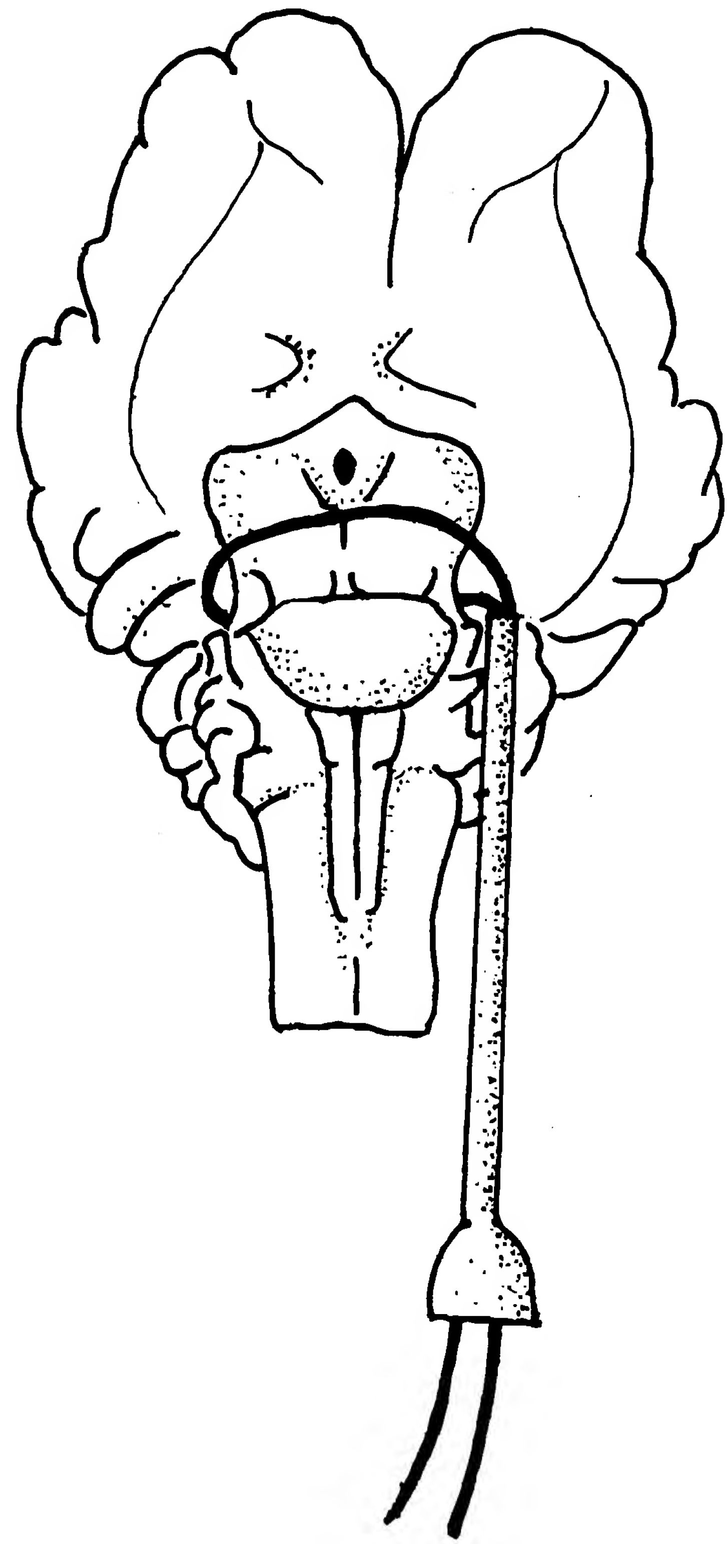

FIG. 1b

Ventral view of bovine brain showing wire loop in place

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HISTOLOGICAL PROCESSING PROCEDURES FOR BRAIN TISSUE FROM SUSPECT BSE CASES

Three separate schedules are described for:

A. Extended processing schedule for large and thicker blocks than standard in an enclosed tissue processor
B. Routine overnight processing schedule using a "Shandon Hypercentre" enclosed tissue processor
C. Processing schedule for a carousel-type tissue processor

A. EXTENDED PROCESSING SCHEDULE FOR LARGE AND THICKER BLOCKS THAN STANDARD IN AN ENCLOSED TISSUE PROCESSOR

Alcohol	70%	2 hours
	70%	3 hours
	90%	6 hours
	90%	6 hours
	100%	3 hours
	100%	3 hours
	100%	6 hours
Chloroform	I	6 hours
	II	6 hours
	III	12 hours
Wax	I (62°C)	8 hours
	II (60°C)	8 hours
	TOTAL	69 hours

Rinse tissues in 70% alcohol before placing on the processor.
Reagents should be changed frequently – after three runs would be a guideline.

B. ROUTINE OVERNIGHT PROCESSING SCHEDULE USING A "SHANDON HYPERCENTRE" ENCLOSED TISSUE PROCESSOR

Alcohol	70%	I	3 hours		room temperature
		II	30 minutes		"
		III	45 minutes		"
Alcohol	90%	I	1 hour	vacuum	"
		II	1 hour	"	"
Alcohol	100%	I	1 hour	"	"
		II	1 hour	"	"
		III	1 hour	"	"
Chloroform		I	2 hours 30 minutes	"	"
		II	3 hours	"	"
Wax M.P.	58°C	I	1 hour 30 minutes	"	62°C
		II	3 hours	"	60°C
	Total		19 hours 15 minutes		

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C. PROCESSING SCHEDULE FOR A CAROUSEL-TYPE TISSUE PROCESSOR

Alcohol	70%	2 × 30 minutes
	90%	2 × 30 minutes
	100%	3 × 1 hour
Chloroform	I	1 × 1 hour
	II	14 hours
Wax	I	2 hours
	II	1 hour
	III	1 hour

Rinse tissues in 70% alcohol before placing on the processor.

The time in chloroform should be adjusted to the maximum that the processor will accomodate within the range 12-18 hours.

Reagents should be changed frequently – after three runs would be a guideline.

STAINING: HAEMATOXYLIN AND EOSIN SCHEDULE

1. De-wax sections in xylene	2 × 2 minutes
2. Absolute alcohol	2 × 1 minutes
3. 70% alcohol	30 seconds
4. Tap water	2 minutes
5. Harris' haematoxylin acidified[a]	8 minutes*
6. Rinse in tap water[b]	2 minutes
7. Acid alcohol (0.1% hydrochloric acid)	10 seconds
8. Wash in tap water[b]	5 minutes
9. "Blue" in saturated (1.5%) lithium carbonate	30 seconds
10. Wash in tap water[b]	10 minutes
11. Eosin ("yellowish"[c] – 2% in tap water, plus formalin to 0.2%)	4 minutes
12. Running water	5 minutes
13. Absolute alcohol	2 × 2 minutes
14. Xylene	3 × 1 minute
15. Mount in DPX	

Notes

a) Acidified Harris' haematoxylin: CLIN-Tech, 1-2 Faraday Way, London SE18 5TR, United Kingdom

b) Where necessary, use "Scotts Tap-Water Substitute" instead of tap water

c) EOSIN. Yellowish. C. I 45380, BDH/MERCK

* The time in haematoxylin will need to be adjusted to provide the correct intensity of stain

DIAGNOSTIC CRITERIA USED IN THE UNITED KINGDOM

The following guidelines for diagnostic criteria are applied in the United Kingdom:

1. ***Positive***: the presence of vacuolation affecting grey matter neuropil and neuronal perikarya with a systematic and usually bilaterally symmetrical distribution (20).

Note: Neuronal vacuolation in the red nucleus is an incidental finding in cattle brains and its occurrence must be disregarded for the purpose of BSE diagnosis.

2. ***Inconclusive***:

a) inadequate submission of material with poor representation of lesion target sites or severe post-mortem change;

b) vacuolation of the grey matter neuropil and/or neuronal perikarya evident only as a minimal change.

Note: *In all cases where the initial examination is inconclusive, further tissue sampling, processing and examination should be done at the discretion of the pathologist.*

3. ***Negative***:

a) absence of lesions in a range of sections which adequately represent lesion target sites;

b) lesions indicating an alternative neuropathological diagnosis with absence of vacuolar changes in BSE lesion target sites.

Note: *Where clinical signs have strongly suggested BSE, or an alternative diagnosis, further sampling should be undertaken at the discretion of the pathologist to reach a diagnosis.*

4. ***Unresolved***: in a few cases, the pathologist may be undecided and/or unable to classify the histopathology, and may wish to have a second opinion before classifying into one of the other three categories.

***

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